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Methanogenesis and Fine Tailings Waste from Oil Sand Extraction: A Microcosm-Based Laboratory Examination.

by

Fervone M. Holowenko

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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DECEMBER 23, 1999

Abstract

In the past decade, biogenic methane production in the fine tailings pond of Syncrude Canada Ltd. has increased markedly. This study addressed the questions: what started methanogenesis; what are the substrates for methanogenesis; and how long will methanogenesis occur? All the fine tailings produced methane but the organic compounds leading to methanogenesis could not be identified. Phenol, *p*-cresol and *m*-cresol stimulated methanogenesis but *o*-cresol, toluene, phenanthrene, anthracene, hexadecane and naphthenic acids did not. Mixtures of the naphthenic acids were toxic to the consortium. In the fine tailings, methanogens and sulfate-reducing bacteria co-exist but the precise relationship between the two populations could not be determined. Bacterial competition stimulated by the addition of nitrate and sulfate inhibited methanogenesis. Fine tailings samples produced between 0.10 to 0.25 mL CH_4/mL tailings in batch methanogenesis monitored for over a year. Methanogenesis was a finite process and slowed after substrates were depleted.

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If the opportunity to spend numerous hours trapped in a small, overheating room tucked away in the bowels of 4th floor Microbiology preparing, anaerobic medium and countless microcosms (1721 to be exact!), was not rewarding enough, having come into

Phil's lab I have built friendships with helpful coworkers. Debbi Coy, who had the job of training me in anaerobic techniques and GC analysis. I am sure she never knew what she was getting into when she suggested we volunteer together. Debbi's technical training, advice, and support has been integral to the success of this project and her friendship has kept me sane during the endless GC runs and the frustrating hours at the computer writing my thesis. Thank you Debbi.

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List of Abbreviations

CT	nonsegregating tailings known as consolidated tailings at Suncor Inc. and composite tailings at Syncrude Canada Ltd.
DOC	dissolved organic carbon
DHNA	decahydro-2-naphthoic acid
dpm	disintegrations per minute
ESIMS	electrospray ionization mass spectrometry
FABMS	fast atom bombardment mass spectrometry
GC	gas chromatograph
GC-EIMS	gas chromatography-electron impact mass spectrometry
GPC	gas proportional counter
HP	Hewlett Packard
MFT	mature fine tailings
MLSB	Mildred Lake Settling Basin
NAs	naphthenic acids
PACs	polycyclic aromatic compounds
SRB	sulfate-reducing bacteria
STP	standard temperature and pressure
TEA	terminal electron acceptor
TOC	total organic carbon

1. Introduction

1.1 A brief overview of the oil sands industry

The presence of oil sands in northeastern Alberta has been documented since the 1780s when surveyors first discovered oil pitch seeping out of the banks of the Athabasca river. Oil sands have been found near Athabasca, Cold Lake, Wabasca and Peace River but the largest deposit, in the Athabasca Basin, has received the most attention. It is estimated that there are over 1.7 trillion barrels of bitumen contained in the Athabasca Basin making it one of the largest reserves of hydrocarbons in the world (MacLean 1998). Of this reserve, 300 billion barrels are recoverable with current technology.

Recognizing the vast potential of the oil sands in the Athabasca region, entrepreneurs in cooperation with the Alberta Government first began mining near Fort McMurray in the early 1920s. Initial attempts failed and after 1949, activity in the oil sands area slowed until the Great Canadian Oil Sands company started mining again in the late 1960s. The company processed its first synthetic barrel of crude oil in 1968 and became Suncor Inc. Syncrude Canada Ltd. followed closely after in 1978.

The oil sands industry has grown dramatically in the last 30 years. In addition to the two successful mines operated by Syncrude Canada Ltd. and Suncor Inc., other companies are now making plans for the development of new mines, while Syncrude Canada Ltd. and Suncor Inc. prepare for further expansion. Currently, the industry produces over 120 million barrels of synthetic crude oil per year but this is expected to increase to 400 million barrels per year within the next 10 years (FTFC 1995a). Syncrude Canada Ltd. is the largest producer of light, sweet crude oil from oil sand and is the largest single source of oil in Canada. Approximately 20 to 25% of Canada's supply of oil comes from the oil sands but as traditional sources for oil continue to be depleted this value is projected to increase to over 50%.

1.1.1 Mining and extraction

A summary of the general process of obtaining bitumen from the oil sands is illustrated in Figure 1.1. The oil sand is located under 10 to 50 m of muskeg soil and overburden (mainly clays) and is obtained by open-pit mining. After the soil and overburden are removed, the oil sand is mined using draglines and bucketwheels or truck and shovel methods. The oil sand is carried from the pit to the extraction plant by truck or a large network of conveyors.

One of the most significant factors determining the success of oil sands operations was the development of a cost-effective means of extracting the bitumen from the sand. Hot water floatation processes had been used but it was the experimental testing and refinements made by Dr. Karl Clark of the Alberta Research Council in the 1920s that led to development of the hot water extraction process used today.

In the Clark Hot Water Extraction, the oil sand is sent from the conveyors to large tumblers where it is digested and conditioned with a mixture of alkaline hot water, caustic soda (NaOH), and steam. The amount of NaOH required for maximum bitumen recovery is dependent on the fines content of the oil sand, as determined by the fraction of solid particles < 44 μm in size (FTFC 1995a). Generally, increasing the fines content increases the amount of NaOH needed for extraction. With the use of NaOH, the pH of the resulting fine tailings range between 8 and 9. At the slurry stage the bitumen separates from the sand. The mixture is then aerated to create a froth to isolate a majority of the bitumen from the sand and clay particles. From the tumblers, the slurry is pumped into primary separation vessels, and diluted in hot water. The froth containing the bitumen floats to the surface of the large separation vessels, while residual sand settles out and is removed. Middlings (slurry located in the central region of the separation vessel) are removed, and further processed to recover any oil droplets which do not separate with the froth. After the water and fine solids are removed from the froth, the bitumen undergoes various stages of

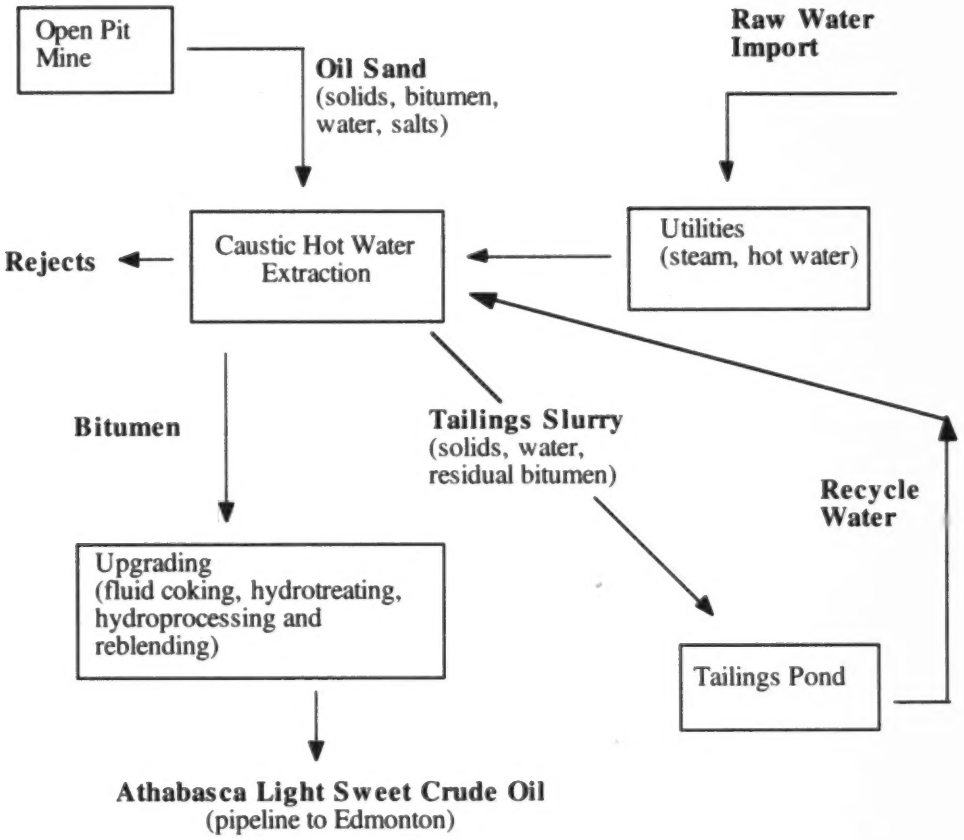


Figure 1.1: General scheme for the processing of oil sand (FTFC 1995a).

upgrading where it is converted to light synthetic crude oil (Figure 1.1). Each day, Syncrude Canada Ltd. processes over 500 thousand tonnes of oil sand (List and Lord 1997) and recovers up to 90% of the bitumen present, producing 250 thousand barrels of bitumen (diluted) which is upgraded to 220 thousand barrels crude oil (MacLean 1998).

1.1.2 Tailings disposal

Every m^3 of mined oil sand requires up to 3 m^3 water and produces on average 4 m^3 of waste. The tailings slurry waste consists mainly of solids (sand and clays), process water, organics and residual bitumen (0.1 to 0.2 g by weight of the solids) (Gulley and MacKinnon 1993; List and Lord 1997). Approximately 1 m^3 of each 4 m^3 of waste is tailings sand which settles out and is used to create dykes and sand beaches (List and Lord 1997). The remaining 3 m^3 consists of water (2.75 m^3) and fine tailings (0.25 m^3 of solids < 44 μm). Due to a "zero discharge" policy, Syncrude Canada Ltd. and Suncor Inc., do not release any extraction wastes from their leases. Consequently, all fine tailings are contained on site, primarily in large tailings ponds. Suncor Inc. has five tailings ponds, covering a total of 16 km^2 and containing over 100 x 10^6 m^3 of fine tailings (Dr. D. Scott, University of Alberta, personal communication). Syncrude Canada Ltd. has several settling basins, the Mildred Lake Settling Basin (MLSB) which covers over 25 km^2, the South West Sand Storage which covers 30 km^2, and the Base Mine Lake (West InPit Storage Pond) which covers about 6 km^2. At present, there is excess of 300 x 10^6 m^3 fine tailings contained in these ponds (FTFC 1995c; List and Lord 1997, M. MacKinnon, personal communication). With current processes and the predicted expansion of the mining operations, the volume of fine tailings could exceed 1 billion m^3 by the year 2030 (FTFC 1995c; Gulley and MacKinnon 1997). In the ponds, the fine tailings fraction undergoes a slow process of sedimentation and consolidation. The resulting ponds have an upper layer of clear water that has been released during consolidation of the fine tailings. This water is recycled back to the plant (Figure 1.1), providing 75% of the water needed for extraction operations (MacKinnon 1989). Under the water layer is a transition zone, which contains a

suspension of diluted fine tailings slurry. With depth, the fine tailings becomes denser and more viscous. As the fine tailings in this zone, consolidate they are considered mature fine tailings (MFT).

The oil sands' tailings water is toxic to aquatic organisms, and the primary group of compounds responsible for the toxicity are naphthenic acids (NAs) (CEATAG 1998; FTFC 1995a). NAs are a natural component of bitumen which are released and concentrated during the process of bitumen extraction. Concentrations of NAs in the tailings pond surface water range between 80 to 120 mg/L. Little is known about these compounds, but they consist of a complex mixture of monocyclic and polycyclic alkanes that contain carboxylated side chains (Figure 1.2). The number of rings in the structure can vary as can the length of the carboxylated side chain as denoted by the subscript q. Little is known about the R group, but it is considered to be aliphatic. NAs have a general formula $C_nH_{2n+z}O_2$ and are classified into Z groups, which represent the number of hydrogen atoms lost as the structures become more compact (CEATAG 1998). NAs with one ring belong to the Z= -2 group, two ringed structures to the Z = -4 group and so on. NAs classified in the Z = 0 family lack a ring structure and therefore do not conform to the definition of a NA. Furthermore, straight chain carboxylic acids are generally classified as fatty acids. Analysis of tailings ponds extracts by fast atom bombardment mass spectrometry (FABMS) found 40 to 50 ions that represented NAs with 0, 1, 2, and 3 rings corresponding to Z values of 0, -2, -4 and -6, respectively (Morales et al. 1993).

1.1.3 Reclamation of the fine tailings

A variety of options for the remediation of the fine tailings are being investigated including a dry landscape approach, a wet landscape approach and most recently, composite or consolidated tailings (FTFC 1995b; List and Lord 1997). Any reclamation option must meet a variety of requirements (Gulley and MacKinnon 1993; FTFC 1995b) ensuring that:

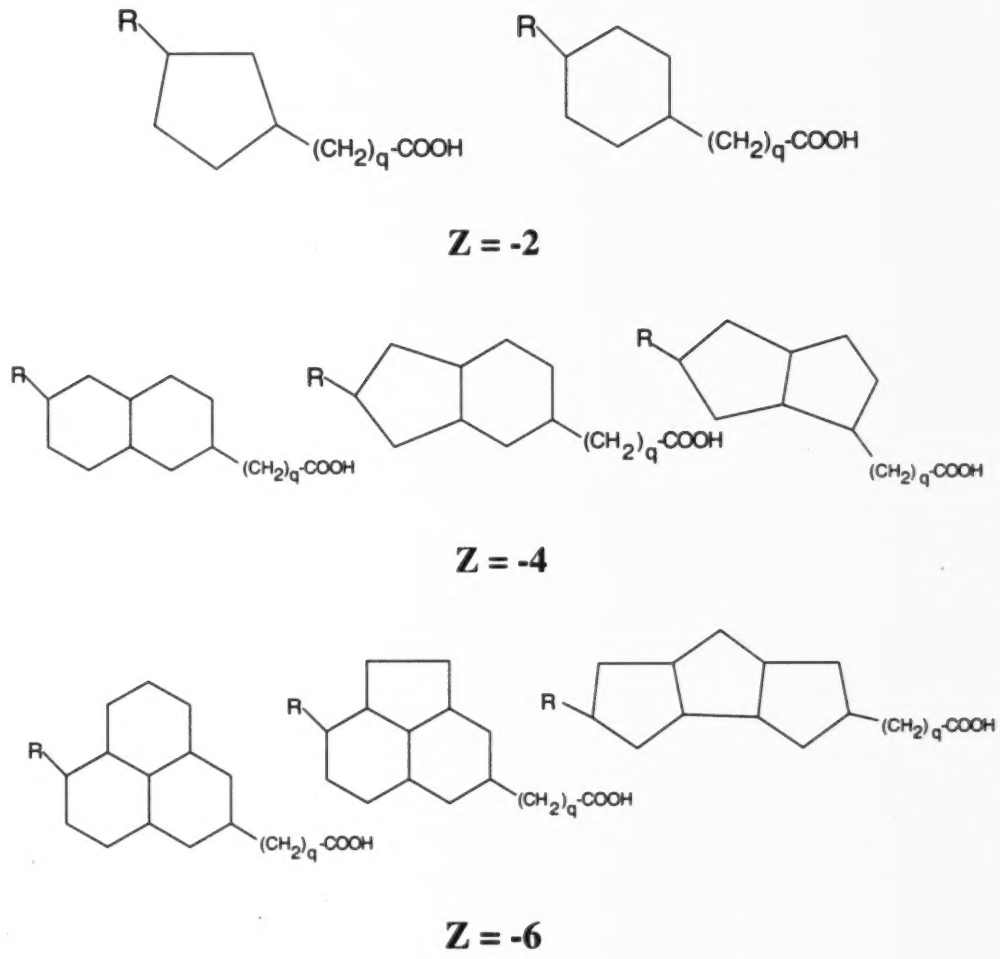


Figure 1.2: Naphthenic acid structures and Z families (from Morales et al. 1993). The subscript q is greater than or equal to one.

1. there is restriction of direct contact or release of contaminants into the environment;
2. there is restriction of off-site transport of contaminants by seepage;
3. there is restriction of hydrological impacts on the fine tailings deposits;
4. the landscape is stable; and
5. the landscape is productive and self-sustaining.

One of the major avenues being pursued for remediating the large volume of fine tailings waste is a wet landscape approach (Gulley and MacKinnon 1993; List and Lord 1997). With this approach, the fine tailings would be transferred from the MLSB into an abandoned mined-out pit, over which a layer of water would be placed, creating a water-capped lake (Boerger et al. 1992; MacKinnon and Boerger 1991). The depth of the water cap must be thick enough to isolate the fine tailings and prevent sediment suspension to create a viable and self-sustaining lake ecosystem (Lawrence et al. 1991). In this productive lake ecosystem, the fine tailings would form the sediment and be covered with detritus, which would minimize mixing of the fine tailings with the water cap and create a biologically active zone. Furthermore, in time, the microorganisms in the lake would degrade any organic acids and other compounds moving up from the fine tailings layer thereby detoxifying the waste (Gulley and MacKinnon 1993). However, for this approach to work, the tailings must be mature, that is they must have a low permeability, high density, high yielding strength and high viscosity. These properties would ensure that a well-stratified ecosystem would develop (FTFC 1995b).

1.1.4 Methanogenesis and the MLSB

The MLSB was established in 1978 when Syncrude Canada Ltd. began operations. It contains three environments that can be classified as aerobic (water layer), anoxic (water:fine tailings interface) and anaerobic (fine tailings layer). The anaerobic layer is the largest and provides a vast volume in which anaerobes can live. Enumeration of anaerobic microorganisms in the MLSB in the early 1990s detected nitrate-reducers, iron-reducers

and sulfate-reducers; methanogens were below detectable limits using the 5-tube MPN assay (Sobolewski 1992). Over the next five years, visible bubbling activity was noted on the surface of the MLSB. Gas bubbles, produced in the fine tailings, were percolating to the surface and erupting (Figure 1.3). Bubbles were first noted primarily on the south side of the MLSB, but soon spread across the whole basin. Analysis of the gas evolving from the MLSB determined that a significant proportion was methane (M. MacKinnon, personal communication). Enumeration of fine tailings samples collected in 1996 detected nitrate-, iron- and sulfate-reducers. This time methanogens were also detected at various locations in MLSB and at depths ranging from 5 to 25 m, with MPN values as high as 4.3 x 10^6/g tailings (dry weight) (Sobolewski 1999).

1.2 Methanogens and methanogenesis

Methanogens are a unique group of prokaryotic microorganisms that are able to respire under anaerobic conditions to produce methane gas. They are the strictest known anaerobes, requiring redox potentials more negative than -300 mV, which corresponds to theoretical O_2 concentrations less than 10^{-56} molecules/L (10^{-80} mol/L) (Hungate 1967) when O_2 is the only factor contributing to the redox potential. Oxygen is harmful to methanogens because it lowers the adenylate charge in the cell, depriving the cell of energy (Roberton and Wolfe 1970) and causes irreversible dissociation of enzyme complexes needed for respiration (Cheeseman et al. 1972; Schönheit et al. 1981). Methanogens have a pH optimum generally near 7 but have been documented in natural settings at pH 3.9 up to 9 (Zinder 1993). They can survive in temperatures ranging between 5 to 110°C and from freshwater to brine environments. They are found in all types of anaerobic environments including gastrointestinal tracts of ruminants and humans, hot springs, hydrothermal vents, sewage digestors, landfill sites, flooded soils, rice paddies, aquatic sediments (freshwater and marine) and the hindgut of termites. While methanogens cannot grow or produce methane in the presence of O_2, there is evidence that they can tolerate exposure to oxygen (Zehnder and Stumm 1988; Zinder 1993).

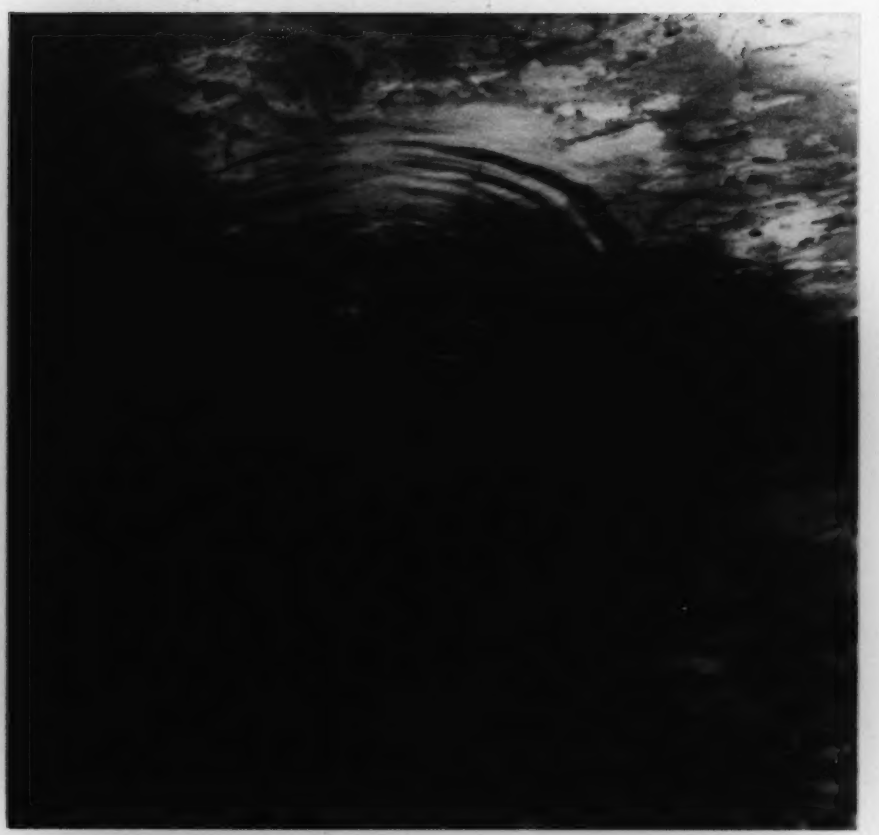


Figure 1.3: Gas bubbles erupting at the surface of the MLSB.

Methanogens are a morphologically diverse group with members consisting of short or long rods, spirilla, and cocci, arranged into long chains or aggregates. Some methanogenic species are motile while others are not, and some form gas vacuoles, while others do not. Despite dissimilar morphologies, methanogens share common features which set them apart from other microorganisms and from Bacteria. Prior to 1974, methanogens were classified as Bacteria based solely on morphological criteria, however physiological studies caused Bryant (1974) to reclassify methanogens into a single group. Later 16S rRNA sequencing (Balch et al. 1979; Woese and Fox 1977) would confirm the unity of methanogens and their relatedness to established members of the kingdom *Archaeobacteria*, known as Archaea, including some extreme halophiles and thermophilic sulfur-dependent organisms.

In addition to their novel rRNA sequences, methanogens have unique lipids, cell walls and co-factors, which set them apart from other prokaryotes (Boone et al. 1993). Unlike eubacteria which have ester-linked lipids, methanogen membranes are composed of isoprenoid ether lipids generally linked to glycerol (De Rosa and Gambacorta 1988; Jones et al. 1987). Furthermore, the glycerol moiety has a stereochemical configuration different in methanogens than in eubacteria (Jones et al. 1987). Methanogens lack murein, the peptidoglycan containing muramic acid that is found in eubacteria cell walls (Kandler and Hippe 1977), and therefore are insensitive to the antibiotics that block peptidoglycan synthesis in eubacteria, including penicillin, cycloserine and valinomycin (Hilpert et al. 1981). Methanogens have at least six unique proteins and co-factors involved in respiration and the production of methane (Jones et al. 1987; Rouvière and Wolfe 1988). Included in this group is factor 420 (F_{420}) which is involved in two electron transfer reactions (Cheeseman et al. 1972) and 2-mercaptoethane sulfonic acid (coenzyme M or CoM) which is the methyl group carrier in the methanogenic pathway (Taylor et al. 1974). Complete genomic sequencing of *Methanococcus jannaschii* has identified the genes for all the known enzymes and enzyme complexes associated with methanogenesis (Bult et al. 1996).

1.2.1 Substrates for methanogenesis

Methanogens have two pathways by which they can generate methane. One is the reduction of CO_2 using H_2 as an electron donor and the second is the reduction of a methyl group. CO_2 reduction is the preferred pathway of methanogenesis for most methanogens and for some it is the only pathway (Whitman et al. 1991). H_2 provides the electrons required for the reduction of CO_2 while water provides the protons (Daniels et al. 1980). H_2 is often provided as a by-product of fermentation activities of anaerobic bacteria, fungi and protozoa. However, formate (Boone et al. 1989; Thiele and Zeikus 1988) and in some cases secondary alcohols such as 2-propanol (Widdel 1986), can serve as electron donors.

Methanogens that form methane by the reduction of a methyl group can be divided into methylotrophic and aceticlastic methanogens. Methylotrophs transfer a methyl group to a carrier which through a variety of steps is reduced to methane. Compounds such as methanol, trimethylamine, and dimethylsulfide are degraded in this manner (Zinder 1993). Electrons for this process come from the oxidation of a portion of the methyl groups to CO_2 or by using available H_2. Aceticlastic methanogens also reduce a methyl group, but in this case, the substrate is both the electron donor and the electron acceptor. Aceticlastic methanogens break the carbon-carbon bond in acetate, oxidizing the carboxyl group to CO_2 to generate electrons and reducing the methyl group to methane to generate energy (Pine and Barker 1956). In certain habitats, aceticlastic methanogens are responsible for a majority of the methanogenesis occurring. For example, over 70% of the methane formed in digested sludge and some freshwater lake sediments is from the mineralization of acetate to methane (Cappenberg 1974; Jeris and McCarthy 1965; Phelps and Zeikus 1984).

1.3 Anaerobic microbial interactions

1.3.1 Microbial hierarchy

Methanogenesis is the terminal step in carbon flow in many anaerobic habitats, because methanogens have a limited substrate range and obtain relatively little energy from the substrates they metabolize. Consequently, methanogens are dependent on other

organisms for their substrates which creates an intricate microbial hierarchy (Figure 1.4). Large complex polymers including proteins and polysaccharides are broken down by fermentative anaerobes to monomers and oligomers including sugars, amino acids, and peptides. These can be further degraded by the same or other anaerobic bacteria to long-chain fatty acids, CO_2 and H_2 or acetate for the methanogens.

1.3.2 Competition

Chemotrophic organisms obtain energy by breaking down usable substrates, a process which produces ATP and reducing equivalents. Most of the potential energy is trapped in the electron carriers and needs to be released (Thauer et al. 1977). In aerobic systems, electrons are released through the electron transport chain with O_2 as the terminal electron acceptor (TEA). Since anaerobic environments lack O_2, reducing equivalents are consumed through fermentation in which an organic compound serves as the TEA or through anaerobic respiration in which an inorganic ion serves as the TEA (Holland et al. 1987; Thauer et al. 1977). Anaerobically-respiring microorganisms are generally classified by the type of TEA they use; hence nitrate-reducers use nitrate as a TEA, sulfate-reducers use sulfate, etc. The presence or absence of a given TEA will dictate the success of an organism to thrive and out-compete other organisms for available electron donors (Zehnder and Stumm 1988; Zinder 1993). Therefore, a hierarchy of competition based on thermodynamic considerations is established. For example, given a mole of acetate, the nitrate-reducers would out-compete the manganese-reducers, which would out-compete the iron-reducers and so on (Figure 1.5), as each higher group is able to obtain more energy from the substrate than the group(s) below it (Holland et al. 1987; Thauer et al. 1977). Methanogens obtain the least amount of energy for a given substrate and therefore make poor competitors (Table 1.1). Consequently, when an environment turns methanogenic, as with the MLSB, it is likely that the TEAs for other anaerobic bacteria have been depleted enough to allow the methanogens to successfully compete for substrates (Holland et al. 1987; Large 1983).

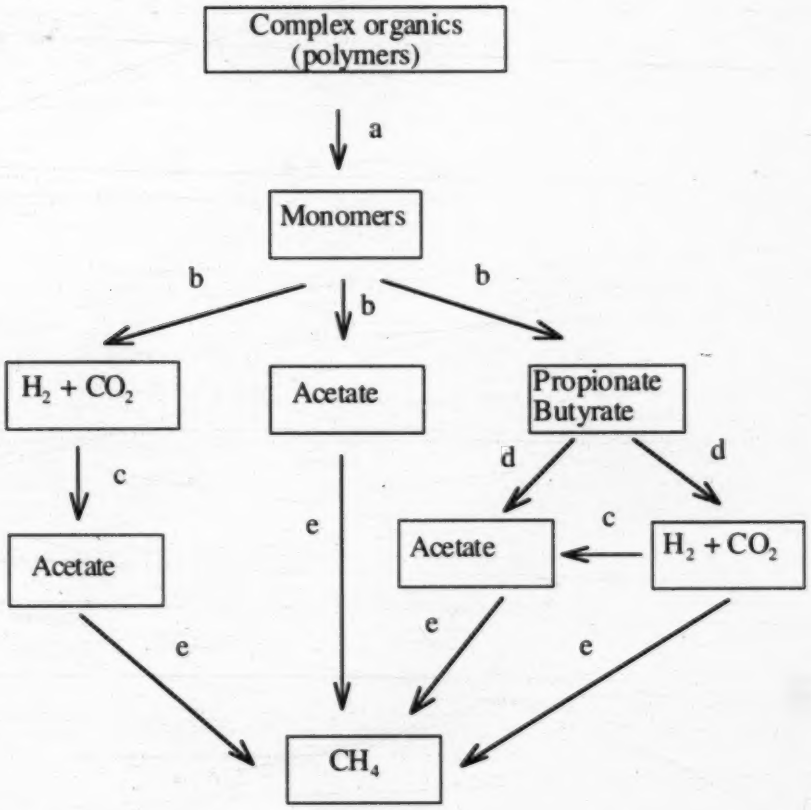


Figure 1.4: Flow diagram of catabolic reactions leading to the formation of methanogenic substrates (Holland et al. 1987; Large 1983; Zehnder and Stumm 1988). "a" represents hydrolysis reactions performed by cellulolytic and hydrolytic bacteria. "b" represents primary fermentation reactions performed by fermentative bacteria. "c" represents acetogenesis accomplished by acetogens. "d" represents a second stage of fermentations performed by fatty-acid oxidizing bacteria (syntrophs). "e" represents methanogenesis performed by methanogens.

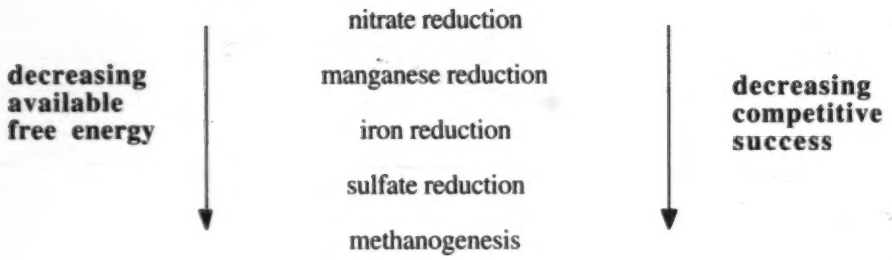


Figure 1.5: Flow diagram of the thermodynamic favorability for substrate utilization within anaerobic environments.

Table 1.1: Comparison of energy obtained from the utilization of H_2 or acetate by iron-reducers, sulfate-reducers and methanogens (Zinder 1993).

Electron Donor	Reaction	$\Delta G^{\circ\prime}$ (kJ)[a]
H_2	$4H_2 + 8Fe^{3+}$ ----------> $8Fe^{2+} + 8H^+$	-914
	$4H_2 + SO_4^{2-} + H^+$ ----------> $HS^- + 4H_2O$	-152
	$4H_2 + HCO_3^- + H^+$ ----------> $CH_4 + 3H_2O$	-135
Acetate	$CH_3COO^- + 8Fe^{3+} + 4H_2O$ ---> $8Fe^{2+} + 2HCO_3^- + 9H^+$	-809
	$CH_3COO^- + SO_4^{2-}$ ----------> $HS^- + 2HCO_3^-$	-47
	$CH_3COO^- + H_2O$ ----------> $CH_4 + HCO_3^-$	-31

[a]values are from Thauer et al. (1977)

The main competitors with methanogens are sulfate-reducing bacteria (SRB), but acetogens and ferric (Fe^{3+}) iron-reducers (Zinder 1993) (Table 1.1) also compete. SRB use sulfate and other oxidized forms of sulfur including thiosulfate, sulfite, and elemental sulfur as TEAs. SRB have a broader substrate range than methanogens including organic acids, alcohols, amino acids, aromatic and linear organic compounds. Acetogens metabolize sugars, purines, methoxyl groups or methoxylated aromatics (Ljungdahl 1986) and compete for H_2. The iron-reducers have not been as well characterized. Initial studies with GS-15, an isolated iron-reducer, demonstrated metabolism of acetate, butyrate, and ethanol (Lovley et al. 1988) under iron-reducing conditions. Later studies showed utilization of toluene, phenol and *p*-cresol (Lovley and Longergan 1990). Pure culture work with the iron-reducer, *Shewanella (Alteromonas) putrefaciens*, showed growth on H_2, formate, lactate and pyruvate (Lovley et al. 1989). Iron-reducing environmental samples can degrade benzene (Kazumi et al. 1997) and toluene (Lovely et al. 1994).

Competition for H_2 is dependent on the partial pressure of H_2 in the habitat. H_2 is generally produced and immediately consumed in anaerobic environments so that there is a low steady-state concentration established. H_2 concentrations *in situ* have been measured in the nM range (Conrad et al. 1986). Methanogens need a H_2 partial pressure of at least 6 x 10^{-5} atm (at 35°C) before they are able to use it as an electron source (Zinder 1993). SRB are able to utilize H_2 more efficiently than methanogens and at a lower threshold, thereby maintaining concentrations below that required by methanogens. Methanogens can utilize H_2 at a lower partial pressure then the acetogens. The role of iron-reducers in H_2 competition is highly dependent on the availablility of Fe^{3+}. If Fe^{3+} is present, H_2 will be consumed by the iron-reducers because of favorable thermodynamics (Figure 1.5) which will reduce H_2 to levels below the threshold for either SRB or methanogens (Zinder 1993).

Competition for acetate is not as complicated as for H_2. The presence of Fe^{3+} or sulfate will favor acetate-utilization by the iron- and sulfate-reducers without a minimum threshold concentration, however, kinetic studies have shown that SRB do have a higher

substrate affinity (lower K_m) for acetate than methanogens (Schönheit et al. 1982). Having a lower K_m value enables the SRB to outgrow the methanogens in the presence of acetate. Methanogens have minimum acetate threshold concentrations which are species-dependent and range from 0.005 to 1.2 mM acetate (Jetten et al. 1990). Higher acetate concentrations favor faster growing methanogenic species (*Methanosarcina*) whereas low acetate concentrations favor slow growing species (*Methanothrix*). *Methanothrix* is a slow growing specialist that only uses acetate. Acetate consumption is slow with low energy yields and only occurs at low acetate concentrations as the acetate K_m value for *Methanothrix* is <1.0 mM. *Methanosarcina* is a fast growing generalist able to grow on many substrates with high energy yields. *Methanosarcina* can only use acetate at higher concentrations because of an acetate K_m value of 3 to 5 mM (Zinder 1993). Regardless of threshold concentrations and affinities, environmental conditions play a role in competition for substrates, but this is poorly understood and cannot be predicted (Zinder 1993).

1.3.3 Synergism

Synergism is another significant microbial relationship occurring within anaerobic environments. Methanogens rely on a hierarchy of organisms to break down large compounds to CO_2 and H_2, and acetate (Figure 1.4); this requires a delicate balance of synergism and competition. However, methanogens are not the only organisms to benefit from this synergism. In some cases, the hierarchy would fail to function if the methanogens were not there to remove the H_2 and acetate by-products formed by the other microorganisms in processes known as interspecies H_2 (or acetate) transfer.

Some of the earliest studies with methanogenic organisms was with a presumably pure culture of *Methanobacillus omelianskii* which was "isolated" in the 1930s. With the development of more sophisticated anaerobic methods, Bryant et al. (1967) determined that the culture of *M. omelianskii* was actually a symbiotic relationship between two organisms, the methanogen (eventually named *Methanobacterium bryantii*) and organism S. Further studies of this symbiotic relationship revealed that the *M. bryantii* was a H_2-consuming

methanogen whose presence was required by organism S to metabolize ethanol. The oxidation of ethanol is a thermodynamically unfavorable reaction (equation [1.1]) however, the methanogen drives the oxidation of ethanol by removing the H_2 (equation [1.2]). This relationship led to the concept of interspecies H_2 transfer (Wolin and Miller 1982).

organism S $CH_3CH_2OH + H_2O \longrightarrow 2H_2 + CH_3COOH$ equation [1.1]

methanogen $CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$ equation [1.2]

Since this first example of interspecies H_2 transfer was understood, other such relationships have been discovered. For example, *Syntrophomonas wolfei* cannot oxidize butyrate without the presence of a H_2-utilizing methanogen (McInerney et al. 1981) and *Syntrophobacter wolinii* cannot degrade propionate without the removal of H_2 by a symbiont (Boone and Bryant 1980). There are other microorganisms which cannot degrade compounds such as benzoate, acetate, stearate, or ethylene glycol without a H_2-consuming methanogen present (Zinder 1993).

While far less studied, the importance of acetate removal by methanogens is being recognized. Removal of acetate helps maintain the pH of anaerobic environments by preventung the build up of acetic acid (Zinder 1993). This is an important aspect of homeostasis and survival of microorganisms. Acetate is a common by-product of many reactions, any increase of its concentration would have a negative effect on the equilibrium. It is beneficial for the acetate-producing organisms to have the acetate removed. However, obligate interspecies acetate transfer has not been reported. Beaty and McInerney (1989) presented initial work with *M. barkerii* and a co-culture of *S. wolfei-Methanospirillum hungatei* and found that butyrate degradation was enhanced in the presence of a acetaticlastic methanogen. Platen and Schink (1987) identified an acetone-degrading culture which contained, an acetone-degrader and a methanogen, and found that the degradation of acetate was a prerequisite for acetone degradation. With continued study it is likely that the role of interspecies acetate transfer in natural environments will be found to be significant.

1.4 The importance of methane

Methane is a colorless, odorless but flammable gas. The carbon atom of methane is in a completely reduced state, unable to receive more electrons. Under anaerobic conditions, methane is very stable but in the presence of oxygen is easily oxidized by aerobic bacteria (methanotrophs) or participates in chemical reactions in the atmosphere (Topp and Pattey 1997). Current methane concentrations in the atmosphere are estimated to be ≈1.7 parts per million by volume (ppmv) (Topp and Pattey 1997; Tyler 1991). Methane is a significant air pollutant and contributes to the greenhouse effect.

Although CO_2 is considered the most important greenhouse gas because of its abundance in the atmosphere (353 ppmv and increasing) (Rodhe 1990), methane is 25 to 30 times more effective than CO_2 in absorbing infrared radiation (Kiehl and Dickinson 1987) which is of particular importance for the greenhouse effect. As radiation from the sun is reflected off the earth it is converted from light radiation to infrared radiation. Molecules in the atmosphere are able to absorb this radiation which traps energy as heat in the atmosphere. Consequently, the temperature of the earth is ≈15 to 30°C higher than it would be if there was no atmosphere (Alexander 1999). The hypothesis of global warming predicts that increasing the concentration of molecules able to absorb infrared radiation will result in more heat being trapped within the atmosphere of the earth eventually leading to an increase in temperature. It has been predicted that if the temperature of the earth were to increase by 1 to 2°C, there would be many consequences (Alexander 1999) such as:

1. ocean levels would rise as seas undergo thermal expansion due to increased temperatures and as glaciers melt;
2. there would be a shift in agriculture as crops would need to be planted further from the equator;
3. species compositions of forests and grasslands would be altered and species would potentially be displaced or eradicated; and
4. wetlands would be lost due to flooding.

While the theoretical impacts of global warming can be debated, there is little doubt that the potential warming of the atmosphere will have significant effects and therefore is of particular concern. Some estimates predict that methane contributes about 15% to the greenhouse effect, less than carbon dioxide at 60% (Rodhe 1990). Others propose that the relative influence of CO_2 is less and that methane contributes two to five times more to the greenhouse gas effect than CO_2 when consideration is given to the concentration of methane in the atmosphere, its absorbing properties and that its destruction produces other greenhouse gases (Tyler 1991). For instance, in the atmosphere, methane reacts to produce CO_2, while methane has a life span of 7 to 10 years, it leads to the production of more CO_2 which takes decades to disappear (Pearman and Fraser 1988; Rodhe 1990).

Methane also participates in numerous chemical reactions in the atmosphere which produce and destroy ozone, O_3 (Tyler 1991). Ozone protects the earth from harmful UV radiation emitted from the sun. The formation of hydroxyl radicals (OH•) from O_3 determines the oxidizing or cleansing efficiency of the troposphere, as OH• is the main oxidant of most gaseous pollutants and maintains the chemical composition of the atmosphere (Lelieveld et al. 1993). Eighty-five to ninety-seven percent of the methane in the atmosphere reacts with OH• (Lelieveld et al. 1993; Pearman and Fraser 1988). Decreasing the OH• concentration lowers the oxidizing power of the atmosphere which prolongs the life of other pollutants which are generally destroyed by OH•. The chemical destruction of methane as it is degraded, is also the major source of CO in the atmosphere (Levy 1973). In addition to being toxic to humans, CO also reacts with OH• to decrease the oxidizing potential of the troposphere and has been found to lower the concentration of O_3 in the atmosphere (Alexander 1999). As both carbon dioxide and methane lower the concentration of OH•, increasing methane concentrations will increase the lifetime of methane as OH• is responsible for the destruction of methane (Lelieveld et al. 1993).

Analysis of gas trapped in polar ice reveals that over the past 100 to 200 years there has been a 2- to 3-fold increase in the concentration of methane in the atmosphere (Khalil

and Rasmussen 1982; Robbins et al. 1973). Presently, methane concentration in the atmosphere is increasing by 0.8 to 2% each year (Khalil and Rasmussen 1982; Lelieveld et al. 1993: Pearman and Fraser 1988) which is largely due to increased anthropogenic sources. However, it is estimated that over half of the methane entering the atmosphere is a result of microbial metabolism (Alexander 1999) and based on a global methane budget, microbial metabolism contributes roughly 350 x 10^9 kg methane each year (Table 1.2).

1.5 Methane and the MLSB

Recent analyses indicate that 2 to 3% of the volume of MLSB is gas and that up to 50% of the gas emitted is methane, the other 50% is primarily composed of CO_2 and N_2. Over 6 km^2 of MLSB is considered an active bubble zone with an estimated flux of 12 g CH_4/m^2/day (M. MacKinnon, Syncrude Canada Ltd., personal communication). With this flux rate indicates the MLSB releases more than 26 x 10^6 kg CH_4 into the atmosphere each year (Figure 1.6). This is 0.0075% of the 350 x 10^9 kg CH_4 released globally by microbial metabolism and is 10% of the total amount of methane produced by Alberta cattle. Of the rumen gas emitted by cows, 40% is methane and on average, one cow produces 200 L CH_4/day (Miller 1991) which results in 52 kg CH_4/year/cow. In one year, the MLSB releases as much methane as 0.5 million cows and the cattle population of Alberta is near 5.5 million (Dr. G. Mathison, University of Alberta, personal communication).

In addition to its role as a greenhouse gas and in atmospheric chemistry, methanogenesis in the MLSB poses potential problems for the remediation of the fine tailings particularly with the wet landscape approach. Since the initiation of gas production in the MLSB, the fine tailings have been consolidating faster than predicted based on chemical and geological considerations (M. MacKinnon, personal communication). Faster consolidation may be beneficial for obtaining dense material to create the foundation of the lake ecosystem but the overall effects of methane formation on the chemical and physical characteristics of the fine tailings cannot be predicted. This may be a problem when trying to anticipate the nature and behavior of the fine tailings in long-term remediation plans.

Table 1.2: Annual release of methane from natural and anthropogenic sources.

Sources	kg x 10^9/year Ref. 1[a]	Ref. 2[b]
Microbial		
Ruminants (livestock)	80 ± 20	80 - 100
Wetlands	130 ± 70	120 - 200
Rice paddies	≈95	70 - 170
Termites	≈10	25 - 150
Landfill	50 ± 25	—[c]
Oceans	10 ± 15	1 - 20
Tundra	—	1 - 5
Other	≈20	23 - 80
Nonmicrobial		
Biomass burning	30 ± 15	10 - 40
Gas & oil production and transport	70 ± 40	10 - 20
Coal mining	35 ± 10	10 - 35
Solid waste	20	5 - 70
Venting and flaring	—	15 - 30
Industrial and pipeline losses	—	15-45
Volcanoes	—	0.5
Automobiles	—	0.5

[a]Lelieveld et al. (1993)
[b]Tyler (1991)
[c]no value reported

Figure 1.6: Eruption of trapped gas at the water surface of MLSB after slight dredging.

Another concern associated with methanogenesis is that after the tailings are capped with water, the methane will continue to percolate into the overlying water re-suspending the fine tailings and moving potentially toxic compounds into the water from the fine tailings sediment (Gulley and MacKinnon 1993). Furthermore, methane in the water could be consumed by aerobic methane-utilizing bacteria (methanotrophs), possibly leading to anoxic conditions. Low oxygen levels could prevent the establishment of an ecosystem with higher forms of life which may compromise the wet landscape approach.

1.6 Objectives of this project

Recognizing the serious implications of methanogenesis in the fine tailings, this project was designed to gain a better understanding of the methanogenic community within the fine tailings, with consideration given to the greater anaerobic consortium present. The major objectives of this project were therefore to:

1. demonstrate methanogenic activity of Syncrude Canada Ltd. fine tailings samples in laboratory microcosms;
2. enumerate methanogens and SRB in Syncrude Canada Ltd. fine tailings samples;
3. enumerate methanotrophs in water samples to see if methane production is increasing the numbers of these methane-consuming bacteria;
4. determine if the onset of methanogenesis was caused by a decrease in SRB activity;
5. determine, in substrate studies, which compounds will yield methane in Syncrude Canada Ltd. fine tailings samples;
6. determine if methane production can be inhibited by the addition of TEAs including sulfate and nitrate;
7. use the data collected to address the following questions:
    a) What started methane production?
    b) What are the potential substrates of methanogenesis?
    c) How long will methanogenesis continue?

2. Methods and Materials

Unless otherwise indicated, all chemicals used were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON) and all media were prepared using double distilled water.

2.1 Sampling and samples

2.1.1 Sample collection

A small boat was used to travel to the various sampling locations on MLSB. To collect each sample, a sterile 4-L glass amber bottle was placed in a weighted harness (Figure 2.1). For sample depths greater than 10 m from the surface of the water, a 30-kg weight was added to the harness to enable the sample bottles to pass through the MFT. The bottle was plugged with a rubber stopper equipped with an eye hook screw attached to a string. The harnessed bottle was lowered to the desired depth and the rubber plug was dislodged. The bottle was considered full when air bubbles ceased to come to the surface of the water. Immediately after the bottle was returned to the surface, subsamples were taken (total volume removed 500 to 600 mL) for pore water and chemical analysis to be done by Syncrude Canada Ltd. personnel. The bottles were capped and transported in ice chests to the University of Alberta where they were stored at 4°C.

When the samples reached the laboratory at the University of Alberta, the headspace of each sample bottle was flushed with O_2-free 30% CO_2 balance N_2 gas to displace any O_2 that may have been present. During subsequent subsampling from these bottles, care was taken to remove samples from below the surface which may have been exposed to O_2 in the headspace during transport.

Due to the thickness and viscosity of the fine tailings samples, subsamples for microbiological studies were obtained with a wide-mouth pipette. Subsamples were removed under a constant stream of O_2-free 30% CO_2 balance N_2 gas.

Figure 2.1: The apparatus used to collect fine tailings samples.

2.1.2 Samples obtained from the Syncrude Canada Ltd. lease (1997 and 1998)

A preliminary set of samples collected on July 15, 1997, were provided by M. MacKinnon of Syncrude Canada Ltd. A water sample from 1 m below the pond surface (M1) and 3 fine tailings samples at depths of 5, 10, and 15 m (M5, M10, M15) were obtained from the middle region of MLSB.

The first major sampling trip took place on August 18 to 20, 1997 at the Syncrude Canada Ltd. lease north of Fort McMurray (Table 2.1). Ten samples were collected from two different areas on MLSB. Site 1 (MB1) was in the central region of the basin and site 2 (MB2) was on the north end. Fine tailings samples increased in viscosity the deeper the sample was collected. Samples were viscous and subsamples contained trapped gas.

The second major sampling trip took place on July 6 to 8, 1998 (Table 2.1). A total of eight samples was collected from two locations in the central region of MLSB. Initially, sampling at MLSB in 1998 was planned for only one location, in the central region of MLSB (MBC1), however, after obtaining samples from 1, 5, and 8 m, penetrating the sampling device through the fine tailings became too difficult. It was decided to move to another location (MBC2) to continue sampling. Due to a limited number of sterile 4-L sample bottles, a water sample was not taken at this second location and only 250 mL of a two fine tailings samples were obtained for enumeration. The second site was approximately 250 to 300 m away from the first. Samples were also collected at these two locations for use by Syncrude Canada Ltd. Unlike my designations (MBC1 and MBC2), no distinction by Syncrude Canada Ltd. personnel was made at these two sites.

At MBC1, there was significant bubble activity on the water surface and the water:tailings interface was between 2 and 3 m below the water surface. The fine tailings were very thick and had a clay-like consistency, thus penetration of the 4-L bottles for sampling was difficult. Some small samples were collected using a new sampler which was designed to hold a 500-mL sampling jar and had a cone base rather than a blunt base which facilitated penetration through the thick fine tailings. At MBC2 there was no visible bubble

Table 2.1: Identification and summary of the samples collected from the Syncrude Canada Ltd. lease in August 1997 and July 1998. Depths were measured from the water surface.

Date	Location	Depth (m)	Designation	Volume (L)	Type
Aug 1997	MLSB Central Region Site 1 (MB1)	1	MB1-1	4	water
		5	MB1-5	4	fine tailings
		10	MB1-10	4	fine tailings
		15	MB1-15	4	fine tailings
		20	MB1-20	4	fine tailings
	MLSB North End Site 2 (MB2)	1	MB2-1	4	water
		5	MB2-5	4	fine tailings
		10	MB2-10	4	fine tailings
		15	MB2-15	4	fine tailings
		20	MB2-20	4	fine tailings
Aug 1997	Base Mine Lake	0.5	MBIP1-1	0.5	water
		5	MBIP1-5	4	fine tailings
Aug 1997	Demonstration Pond	1	DP-1	0.5	water
		5	DP-5	4	fine tailings
July 1998	MLSB Central Region Site 1 (MBC1)	1	MBC1-1	0.5	water
		5	MBC1-5	8	fine tailings
		8	MBC1-8	4	fine tailings
		10	MBC1-10	0.5	fine tailings
	MLSB Central Region Site 2 (MBC2)	5	MBC2-5	4	fine tailings
		10	MBC2-10	0.25	fine tailings
		15	MBC2-15	8	fine tailings
		20	MBC2-20	0.25	fine tailings
July 1998	Base Mine Lake	1	MBWIP-1	0.5	water
		10[a]	MBWIP-10	8	fine tailings
July 1998	Demonstration Pond	1	98DP-1	0.5	water
		5	98DP-5	0.5	fine tailings

[a]see text

activity at the surface of the water and the fine tailings samples were not as thick as those collected from MBC1 and the water:tailings interface was not determined here.

Samples were also obtained from the Demonstration Pond in 1997 (DP) and 1998 (98DP) (Table 2.1). The Demonstration Pond is a small scale wetland created in 1993 as a pilot project of the wet landscape remediation approach. The base of the Demonstration Pond was created with MFT from the MLSB before methanogenesis in the MLSB had become a significant concern. At the time of sampling, the surface water of the pond was clear and plants and sedges were growing in and around the pond. Birds were nesting successfully in the area. The methanogenic activity in the pond was minimal as there was no bubble activity at the water surface. The fine tailings samples collected were fairly fluid and subsamples collected contained little trapped gas. In 1998, the water:tailings interface was about 3 m below the water surface.

Fine tailings samples were also collected from the Base Mine Lake (Table 2.1). The Base Mine Lake is in the preliminary stages of the full-scale wet landscape remediation project and is contained within the old mined-out pit on the Syncrude Canada Ltd. lease. MFT from MLSB is pumped to the Base Mine Lake via pipeline at a rate of over 2.0×10^5 L/min. After the pit has been filled, plans are to continue with remediation by capping the fine tailings with water. The Base Mine Lake consists of 2 separate areas, denoted West InPit 1 and 2. Samples were collected from West InPit 1 in August 1997 (MBIP1) and from West InPit 2 in July 1998 (MBWIP). The bubble activity in the Base Mine Lake was visible. In July 1998, a 5 m sample was not collected because this depth was within the water:tailings interface region which was not the case in August 1997. Collection of the 10 m sample was complicated, as penetration through the fine tailings was difficult. When the empty 4-L bottle was first lowered into the fine tailings, it would not penetrate past 9 m, however, as it began to fill up, the bottle penetrated down to 10 and 11 m and at one point was down 12 m. Therefore the 10 m assignment is an approximate designation (Table 2.1).

2.2 Enumeration of microorganisms

2.2.1 The 5-tube MPN method

The standard 5-tube MPN method (Standard Methods 1985) was employed to enumerate the microorganisms. Ten-fold serial dilutions of the samples were prepared in appropriate dilution blanks. With each successive dilution, the sample was mixed thoroughly to ensure the homogeneous distribution of the sample. MPN tubes containing 9 mL medium were inoculated with 1 mL of a given dilution, so that each dilution was used to inoculate a set of 5 tubes. The tubes were then scored for positive and negative results after the appropriate incubation time. The MPN/mL results were converted to MPN/g dry weight by dividing by the dry weight of the fine tailings samples (g/mL) (Appendix A).

2.2.2 Enumeration of methanogens

A modified anaerobic bicarbonate-buffered medium was prepared by adding 4.0 g NaOH to 1 L boiled H_2O under a constant stream of O_2-free 30% CO_2 balance N_2 gas. Once equilibrated (pH 7.2-7.3), 2.0 g yeast extract (Difco Laboratories, Detroit, IL), 2.0 g trypticase peptones, 0.5 g mercaptoethane sulfonic acid (Jain et al. 1991), 14 mL mineral solution I, 1.4 mL mineral solution II and 14 mL 0.1 g/L resazurin were added (Fedorak and Hrudey 1984). Nine milliliters of medium were dispensed into flushed Hungate tubes (15 mL) (Belco, Vineland, NJ) which were sealed and sterilized by autoclave for 20 min at 121°C. After cooling, filter-sterilized vitamin B solution (0.1 mL) and freshly prepared 2.5% (w/v) $Na_2S{\cdot}9H_2O$ (0.1 mL) were added to each tube.

Mineral solution I contained: 50 g NaCl; 10 g $CaCl_2{\cdot}2H_2O$; 50 g NH_4Cl; 10 g $MgCl_2{\cdot}6H_2O$; 1 L H_2O. Mineral solution II contained: 10 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$; 0.1 g $ZnSO_4{\cdot}H_2O$; 0.3 g H_3BO_3, 1.5 g $FeCl_2{\cdot}4H_2O$; 10 g $CoCl_2{\cdot}6H_2O$; 0.03 g $MnCl_2{\cdot}4H_2O$; 0.03 $NiCl_2{\cdot}6H_2O$; 0.1 g $AlK(SO_4)_2{\cdot}2H_2O$; 1 L H_2O. The vitamin B solution consisted of: 0.1 g nicotinic acid; 0.1 g cyancocobalmine; 0.05 g thiamine; 0.05 g *p*-aminobenzoic acid; 0.25 g pyridoxine; 0.025 g pantothenic acid; 1 L H_2O.

The Hungate tubes were inoculated from a 10-fold dilution series prepared in pre-reduced anaerobic bicarbonate medium (the dilution series was also used to inoculate the SRB MPN tubes, see section 2.2.3). The inoculated tubes were incubated for 3 mon in the dark at room temperature as per Sobolewski (1999). The presence of methanogens was detected by analyzing 0.1 mL headspace gas samples for methane using GC (section 2.9.1). Positive scores were given to tubes which produced more than 0.16% vol CH_4 which was the lower detection limit of the GC.

Both H_2-utilizing and acetate-utilizing methanogens were enumerated. Either 5 mL of H_2 was added to inoculated MPN tubes as an overpressure or acetate was added to the medium for a final concentration of 3 g/L (50 mM) acetate in the tubes.

Methanogens were enumerated in all of the fine tailings samples obtained (July 1997, August 1997 and July 1998). As well, a second enumeration was performed on sample MBIP1-10 (August 1997) after the sample had been stored for 7 mon at 4°C. Replicate enumerations of H_2- and acetate-utilizers at room temperature were performed on two samples collected in July 1998 (MBC1-5 and MBWIP-10).

2.2.3 Enumeration of SRB

Modified Butlin's medium (Butlin et al. 1949) was used to enumerate SRB which supplied lactate as the electron donor. Each liter of water contained: 0.5 g K_2HPO_4; 1 g NH_4Cl; 2 g Na_2SO_4; 0.067 g $CaCl_2 \cdot 2H_2O$; 1 g $MgSO_4 \cdot 7H_2O$; 2.5 mL of 60% sodium lactate; 1 g yeast extract (Difco Laboratories); 0.004 g $FeSO_4 \cdot 7H_2O$ and 10 mL of 0.1 g/L resazurin. The pH was adjusted to 7.5. Test tubes (18 x 150 mm) received two iron finishing nails (rinsed three times with methylene chloride and three times with H_2O) and 9.4 mL medium. The tubes were capped with Kaputs (Fedorak et al. 1987) and sterilized by autoclave for 20 min at 121°C. The tubes were then inoculated using the prepared dilution series (see section 2.2.2) and incubated in the dark at room temperature for 30 days. The cultures were scored for SRB growth as indicated by the formation of a black

iron sulfide (FeS) precipitate on the nails caused by the reaction of iron with sulfide ions (S^{2-}) produced by the reduction of sulfate (SO_4^{2-}) by the SRB.

SRB were enumerated in all of the samples obtained (July 1997, August 1997, and July 1998). Electron donors other than lactate were used to enumerate a subset of the July 1998 samples. The electron donors were added to the modified Butlin's medium lacking lactate in the following amounts (per L): 2.0 g sodium acetate, 0.5 g sodium benzoate, 1.5 g propionic acid or 5 mL H_2 gas added to inoculated tubes as per Collins and Widdel (1986). When H_2 was the electron donor, Hungate tubes rather than test tubes were used.

2.2.4 Enumeration of methanotrophs

A phosphate-buffered mineral salts medium was used (Palumbo et al. 1995). To 1 L of water 0.9 g NaCl; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.5 g $NaNO_3$; 0.044 g $NaH_2PO_4 \cdot H_2O$; 0.26 g K_2HPO_4; 10 mL trace mineral solution; and 1.0 mL ten-fold concentrated vitamin solution was added. The medium was adjusted to pH 7.1. The trace mineral solution contained: 1.0 mg $FeCl_2 \cdot 4H_2O$; 0.5 mg $MgCl_2 \cdot 6H_2O$; 0.11 mg Na_2WO_4; 0.5 mg $MnCl_2 \cdot 4H_2O$; 0.5 mg $CoCl_2 \cdot 6H_2O$; 0.5 mg $CaCl_2 \cdot 2H_2O$; 0.3 mg $ZnCl_2$; 0.01 mg $CuCl_2 \cdot H_2O$; 0.03 mg H_3BO_3; 0.05 mg $Na_2MoO_4 \cdot 2H_2O$; 5.0 mg NaCl; 0.08 mg Na_2SeO_3; 0.1 mg $NiCl_2 \cdot 6H_2O$; 1 L H_2O (Phelps et al. 1994). The vitamin solution contained: 2 mg biotin; 2 mg folic acid; 10 mg pyridoxine HCl; 5 mg riboflavin; 5 mg thiamine; 5 mg nicotinic acid; 5 mg pantothenic acid; 0.1 mg vitamin B_{12}; 5 mg *p*-aminobenzoic acid; 5 mg thiocitic acid; 1 L H_2O (Wolin et al. 1963). Nine milliliters of medium were added to Hungate tubes (20 mL) which were capped and autoclaved for 20 min at 121°C.

Each tube received 1 mL inoculum (from dilution series prepared in the phosphate-buffered mineral salts medium) plus filter-sterilized methane to 5% vol of the headspace. The tubes were incubated for 30 days at room temperature. Microbial growth was determined by turbidity at days 14 and 30 and by monitoring for methane consumption and carbon dioxide production. Water sample M1 (July 1997) was enumerated as were three pure cultures obtained from the American Type Culture Collection (ATCC) which served as

positive controls. The pure cultures used were *Methylomonas methanica* (ATCC 35067), *Methylococcus capsulatus* (ATCC 19069) and *Methylocystis parvus* (ATCC 35066).

2.3 Preparation of anaerobic microcosms

In order to study methanogenesis in the fine tailings samples, serum bottle microcosms were created. Serum bottles ranged in size from 58-mL to 158-mL, but generally 125-mL bottles were employed. All of the microcosms prepared received the anaerobic bicarbonate medium as described in the section 2.2.2. The medium was dispensed into serum bottles flushed with O_2-free 30% CO_2 balance N_2 gas, after which substrate and other supplements were added. The bottles were plugged with butyl rubber stoppers and then placed in an "anaerobic press" (Belco) and autoclaved for 20 min at 121°C. The press was employed to keep the stoppers in the bottles during the sterilization process. After cooling, the bottles received 0.1 mL filter-sterilized vitamin B solution (section 2.2.2) and were reduced using freshly prepared 2.5% (w/v) $Na_2S{\cdot}9H_2O$. The bottles were re-opened and flushed with O_2-free 30% CO_2 balance N_2 gas for inoculation, after which the bottles were plugged with cut butyl rubber stoppers and sealed with aluminum crimps. The butyl rubber stoppers were cut so that the 0.5 inch insulin needles used to collected gas samples for methane analysis could easily penetrate into the microcosm headspace. Microcosms generally received a 1:1 ratio of medium to inoculum. Culture volumes ranged from 20 to 30 mL; headspace volumes ranged from 95 to 105 mL.

The microcosms were incubated in the dark at room temperature or 14°C and were prepared in triplicate unless otherwise indicated. Headspace samples were removed regularly for methane analysis. The presence of resazurin in the medium was a visible indication that the microcosms remained anaerobic throughout the experiment. Having initially been reduced from its pink to colorless form by the addition of $Na_2S{\cdot}9H_2O$, if anaerobic conditions were compromised, the resazurin would return to its pink oxidized form.

2.4 Inhibition of methanogenesis

2.4.1 The effects of mixing active and inactive samples

Sterile 58-mL serum bottles were inoculated with 20 mL of fine tailings. DP-5 was selected as the inactive sample as it had the lowest methane production of all the samples and had the lowest methanogen MPN values (10^4/g). MB1-15 was chosen as the active methanogenic sample as it generated methane and had a large methanogen MPN values (>10^6/g). Three inoculum regimes were used, one using 20 mL of DP-5, another using 20 mL of MB1-15 and a third using 10 mL of each of DP-5 and MB1-15. These three inocula were tested without supplements, with 20 mL H_2-supplement, and with 1000 mg/L acetate-supplement. Each microcosm received sterilized anaerobic resazurin solution so that the final concentration in the microcosms was 1.0 mg/L.

2.4.2 Controlling methanogenesis by the addition of TEAs

2.4.2.1 The effect of nitrate

A preliminary trial with fine tailings samples collected in July 1997 was initiated to study the effect of 3000 mg/L nitrate on methanogenesis. $NaNO_3$ (42 mg) was weighed and added to 58-mL serum bottles to which 5 mL medium and 5 mL inoculum (M5, M10, M15) were added. Unsupplemented microcosms served as controls. Due to the limited amount of sample available, only one microcosm was set up for each treatment.

Following the results obtained from the preliminary study, a larger experiment was conducted using August 1997 samples. Nitrate stock solutions were prepared in concentrations that were 10-fold greater than the desired microcosm concentration and 1-mL portions were added to 58-mL bottles containing 4 mL medium and inoculated with 5 mL of fine tailings samples. Microcosms were incubated at 14°C.

Nitrate was tested at concentrations of 600, 1800 and 3000 mg/L, with $NaNO_3$ as the nitrate source. Control microcosms contained 1 mL of water in place of the nitrate

solution. MB1-5, MB1-15, MB2-5, MB2-15, MBIP1-5, and DP-5 were the August 1997 fine tailings samples used.

2.4.2.2 The effect of sulfate

Experiments similar to those described in section 2.4.2.1 were prepared using sulfate as the TEA added. Following the same protocol, a preliminary experiment tested the effect of 2000 mg/L sulfate on the July 1997 samples. Na_2SO_4 (42 mg) was weighed and added to 58-mL bottles to which 5 mL medium and 5 mL inoculum (M5, M10, M15) were added. Again, due to the limited amount of sample, only one microcosm was set up for each treatment.

Following the preliminary study, a larger experiment was initiated using August 1997 samples. Sulfate was tested at concentrations of 2000, 5000 and 8000 mg/L with Na_2SO_4 as the sulfate source. The microcosms were incubated at 14°C.

2.4.3 Effect of molybdate on methanogenesis

In order to study the effect of adding molybdate to methane-producing microcosms, the following six conditions were studied: no supplements (control); 4400 mg/L (20 mM) molybdate; 1500 mg/L (50 mM) acetate; acetate + molybdate; H_2; and H_2 + molybdate. Serum bottles (125-mL) received medium with supplements (20 mL) and 10 mL inoculum. 50 mL H_2 were injected into the appropriate microcosms as an overpressure.

Heat-killed sterile controls were prepared for each treatment by autoclaving the microcosms for 30 min at 121°C. Five August 1997 samples (MB1-5, MB1-15, MB2-5, MB2-15, and DP-5) were used as inocula. One set of microcosms for each sample was incubated at 14°C and an additional set inoculated with MB1-5 was incubated at room temperature.

In response to some unexpected results noted in the first experiment involving molybdate (see above paragraphs), a second set of microcosms was prepared. August 1997 sample MB1-5, was used to inoculate two series of treatments identical to the first

experiment. One series was incubated at room temperature and the other at 14°C. Sewage sludge collected from the Gold Bar Wastewater Treatment Plant (Edmonton, AB) was also used as an inoculum and microcosms were prepared as described.

2.5 Potential substrates for methanogenesis

One of the original questions to be asked with regards to methanogenesis in the fine tailings samples was, what are the substrates supporting methanogenesis? To address this question, microcosm studies were initiated in which potential substrates were added to fine tailings samples. It was expected that if the added compound was utilized by the consortium, methanogenic substrates would be produced which would stimulate methane production. Comparison of methane (% vol) produced by the microcosms supplemented with the potential methanogenic substrate and the unsupplemented control microcosms would indicate whether the added compound could stimulate methanogenesis, providing insight into which compounds within the complex matrix of the fine tailings could be supporting methanogenesis. A variety of compounds was tested and grouped into the following categories: petroleum, aromatic compounds, and NAs.

2.5.1 Petroleum

2.5.1.1 Potential for methane biogenesis from Prudhoe Bay crude oil

A series of microcosms was established using Prudhoe Bay crude oil as a source of hydrocarbons. Twenty microliters were added to 158-mL bottles containing 50 mL inoculum and 10 mL medium. Control microcosms received 20 µL distilled water. August 1997 samples tested included MB1-10, MB2-10 and MBIP1-5.

2.5.1.2 Potential for methane biogenesis from bitumen

Sterile pre-flushed 58-mL serum bottles received 25 mL inoculum and 3 mL of 10 mg/L sterile, pre-reduced resazurin (final concentration of 1.0 mg/L). In all cases the resazurin retained its reduced form during inoculation and incubation. Before sealing, the

microcosms were supplemented with 250, 750 or 2000 mg oil sand bitumen (provided by M. MacKinnon). To add the bitumen, the desired amount was weighed onto Whatman glass microfiber filters (Coates et al. 1997) in a fume hood using a wooden applicator stick. The surface area of the filters could not hold more than 750 mg each, therefore, some bottles received more than one filter. The filters were pushed through the serum bottle openings and the bottles were stoppered. During addition, some bitumen stuck to the neck walls of the serum bottles. The control microcosms received two clean filters. Each microcosm was shaken vigorously to facilitate the disintegration of the filters. July 1998 samples MBWIP-10 and MBC1-8 were sources of inocula.

2.5.2 Aromatic compounds

2.5.2.1 Potential for methane biogenesis from a mixture of PACs

A preliminary experiment was designed to test whether a variety of PACs could stimulate methanogenesis in the fine tailings samples. To do this, 50 µL of a PAC mixture prepared in diethyl ether were pipetted onto cut squares of Whatman glass microfiber filters and the solvent was allowed to evaporate in the fume hood for at least 3 min, as per Coates et al. (1997). After evaporation the dried filters were added to 158-mL serum bottles containing 50 mL fine tailings inoculum and 10 mL medium. The serum bottles were sealed and shaken to facilitate disintegration of the glass filters, allowing for distribution of the PACs. Control microcosms received filters coated with 50 µL of diethyl ether, which was allowed to evaporate. August 1997 samples tested were MB1-10, MB2-10 and MBIP1-5.

The PAC mixture was prepared in 5 mL diethyl ether and contained ≈300 mg each of naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, dibenzothiophene, phenanthrene, anthracene, fluorene, fluoranthene, biphenyl, and ≈200 mg each of 4-methylbiphenyl and benzothiophene. Addition of 50 µL of the PAC solution caused each microcosm to receive 1.5 mg of each compound. The exception was benzothiophene which has been shown to be inhibitory to aerobic bacteria (Fedorak and Grbic-Galic 1991), only

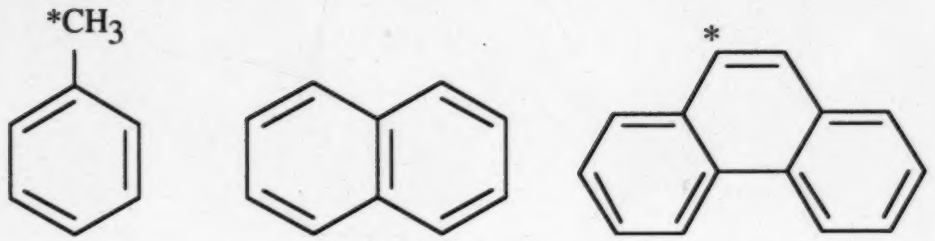
1.0 mg of this compound was added. Anthracene is not very soluble in diethyl ether (Merck 1968) so it was assumed that this compound was the only one that did not dissolve. The PAC mixture was decanted from the undissolved crystals and more diethyl ether was added to the crystals. Some dissolution occurred and GC analysis of the solution showed only one peak which eluted at the same time as the anthracene standard. GC analysis of the PAC mixture indicated that some anthracene was dissolved in the mixture as well.

2.5.2.2 Potential for anaerobic mineralization of ^{14}C-aromatic hydrocarbons

To test whether fine tailings samples could metabolize aromatic hydrocarbons, three compounds were chosen: radiolabelled toluene, naphthalene, and phenanthrene (Figure 2.2). Two sets of positive controls were established using radiolabelled acetate and hexadecanoic acid (Figure 2.3). In addition to the aromatic hydrocarbons tested, hexadecane, a long straight-chain hydrocarbon, was also tested for susceptibility to mineralization. All of the radiochemicals were purchased from Amersham (Oakville, ON) except the toluene which was bought from Sigma Canada Ltd.

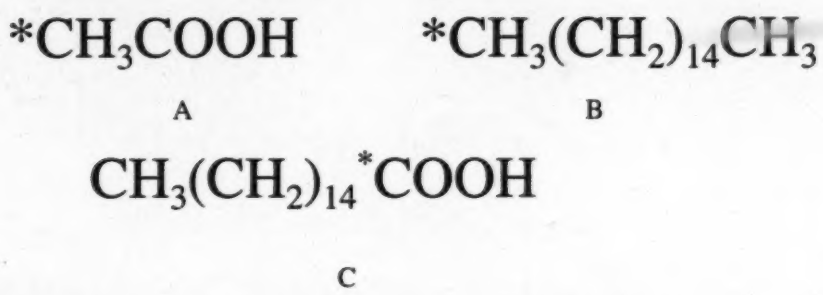
Serum bottles (158-mL) received 75 mL fine tailings inoculum, 10 mL 7.5-fold concentrated medium and a radiolabelled compound (Table 2.2) and were sealed with specially designed composite stoppers (see below). One set of 26 microcosms was incubated under methanogenic conditions, and a second set of 26 microcosms had sulfate added to create sulfate-reducing conditions.

Radioactive stock solutions were prepared in diethyl ether to a concentration of ≈20 μCi/mL as per Coates et al. (1997). Using a 50-μL Hamilton glass syringe, 25-μL aliquots of the radioactive stocks were dispensed onto half circles of Whatman glass microfiber filters for a predicted addition of ≈0.5 μCi (Table 2.2). The diethyl ether was allowed to



A B C

Figure 2.2: Chemical structures of the aromatic hydrocarbons used in ^{14}C-studies. Toluene (A), naphthalene (B) and phenanthrene (C). * denotes position of ^{14}C. Naphthalene was uniformly labelled around the benzene ring.



A B C

Figure 2.3: Chemical structures of the linear compounds used in the ^{14}C-studies. Acetate (A), hexadecane (B), and hexadecanoic acid (C). * denotes position of ^{14}C.

Table 2.2: Summary of the microcosms prepared for the ^{14}C-compound mineralization experiment under both methanogenic and sulfate-reducing conditions.

Compound	Sterile Controls	Replicates	Activity Added (μCi)[a]
2-^{14}C-acetate	1	2	0.50
n-[1-^{14}C]-hexadecanoic acid	1	2	1.75
n-[1-^{14}C]-hexadecane	1	4	0.49
toluene-methyl-^{14}C	1	4	0.41
naphthalene[U-^{14}C]-benzene ring	1	4	0.05
9-^{14}C-phenanthrene	1	4	0.54

[a]the discrepancy between the desired (0.5 μCi) and the actual activity added is a result of the concentration of the original radioactive stock prepared

evaporate for 3 min. The filters were then added to serum bottles containing medium and inoculum. The sealed microcosms were vigorously shaken to facilitate the disintegration of the glass filters and the distribution of the radiolabelled compound.

Heat-killed sterile controls were prepared by adding 75 mL of the appropriate inoculum to a flushed 158-mL serum bottle containing 10 mL medium. The bottles were sealed and autoclaved for 1 h at 121°C on 3 consecutive days. These bottles were re-opened for the addition of the radioactive substrate.

July 1998 fine tailings samples, MBC2-15 and MBWIP-10, were inoculum sources and the production of $^{14}CO_2$ and $^{14}CH_4$ was monitored every 4 to 6 wk, using a ^{14}C trapping system described in section 2.9.9. Sulfate concentrations in the sulfate-supplemented microcosms were also measured. One replicate of each treatment was left unanalyzed as a spare, in case any of the stoppers in the regularly analyzed replicates failed.

Hydrocarbons can adsorb to the butyl rubber stoppers normally used, therefore they could not be used to seal these microcosms. Hydrocarbons adsorb far less to Teflon stoppers, however Teflon is permeable to O_2 and therefore cannot be used for studies with anaerobic microcosms. An understanding of the characteristics of both the butyl rubber and Teflon stoppers led to the construction of composite stoppers using a hybrid of the two types of stoppers (Dr. J. Suflita, University of Oklahoma, personal communication). To create the hybrid stopper, the top half of a cut butyl rubber stopper was glued to a bottom half of a cut Teflon stopper using a toluene-based contact cement. Once cemented together the composite stopper can be autoclaved prior to use.

2.5.2.3 Potential for methane biogenesis from phenols

2.5.2.3.1 Studies with August 1997 fine tailings samples

Microcosms were established with 150 mg/L phenol, *m*-cresol, *p*-cresol or *o*-cresol (Figure 2.4). Serum bottles (125-mL) received 10 mL fine tailings, 8 mL medium, and 2 mL of either H_2O (control) or the substrate stock solutions (treatments). Substrate stock

solutions were prepared at 1500 mg/L. Phenolic concentration and methane production were monitored over time. When the concentration of any of the phenolic compounds tested was depleted, the microcosms were re-supplemented with 2 mL of the original stock solution replenishing the substrate level back to near 150 mg/L. According to Buswell's equation (section 2.11.1), 150 mg/L phenol will produce 2.5 mL of methane at STP when completely mineralized while the cresols will produce 2.6 mL of methane at STP.

Six of the August 1997 fine tailings samples were tested including MB1-5, MB1-15, MB2-5, MB2-15, MBIP1-5 and DP-5. Microcosms were incubated at room temperature. Two additional sets of microcosms containing samples MBIP1-5 and DP-5 were incubated at 14°C. At the time of inoculation, the fine tailings samples had been stored at 4°C for over 8 mon.

2.5.2.3.2 Studies with July 1998 fine tailings samples

In 1998, a repeat phenolic substrate experiment was established using 10 day old samples. In addition to phenol and the three cresols treatments, a positive control was established in which microcosms received 375 mg/L acetate. Mineralization of 375 mg/L acetate will result in the production of 2.5 mL methane. Samples MBC1-5, MBC1-8, MBC2-5, MBC2-15, and MBWIP-10 were tested and incubated at room temperature and a second set of microcosms with samples MBC2-5 and MBC2-15 was incubated at 14°C.

2.5.3 Naphthenic acids

2.5.3.1 Potential for methane biogenesis from commercial NAs in sewage sludge

Commercial NAs (Kodak P9513) at concentrations of 50, 200, 500, 1000, 2000 mg/L were tested for their ability to stimulate methanogenesis in sewage sludge. Serum bottles received 2 mL of NAs solutions, 8 mL medium and 10 mL inoculum. Sewage sludge was collected from Gold Bar Wastewater Treatment Plant (Edmonton, AB) on June 2, 1997 was used as an inoculum. Microcosms were incubated in the dark at 37°C.

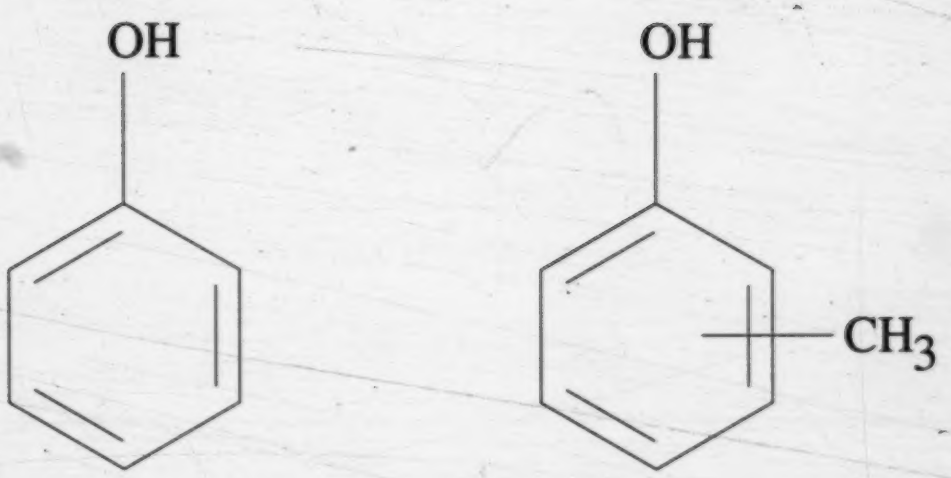


Figure 2.4: The chemical structures of phenol (A) and cresol (B). There are three cresol isomers depending on the position of the methyl group.

2.5.3.2 NAs extracted from the MLSB

2.5.3.2.1 Extraction of NAs

Tailings water from MLSB was collected on July 15, 1997 and from it the naturally occurring NAs were extracted. The water (160 L) which contains the sodium salts of the NAs was acidified with H_2SO_4 to pH 2-3 to precipitate acids out of solution and was allowed to settle for a week. After most of the suspended solids had settled, the water was siphoned off until ≈750 to 1000 mL remained. After most of the water had been removed by siphoning, the remaining liquid which consisted primarily of the settled solids (organic acids) was transferred into 250-mL centrifuge bottles and centrifuged at 14500 rpm for 15 min (IECB-22M programmable centrifuge at 20°C). The supernatant was decanted and discarded and the process was repeated. The pellets were then rinsed with 0.1 N NaOH to ionize the NAs and shaken for 15 min followed by 15 min of centrifugation. The brown-blackish supernatant containing the NAs was decanted into a stock jar. The pellets were washed twice with 0.1 M NaOH to ensure adequate extraction of the acids present. The concentration of the NAs in the stock solution was determined via extraction into methylene chloride and FT-IR spectroscopy (Jivraj et al. 1995) by B. Fung of Syncrude Canada Ltd. Just over a liter of stock NAs was collected at a concentration of 3100 mg/L (pH 11.8) and was stored at 4°C to be used in subsequent experiments.

2.5.3.2.2 Potential for methane biogenesis from extracted NAs

In order to study the effect of different concentrations of extracted NAs on methane production, the following six treatments were prepared: 0 mg/L extracted NAs (control), 50 mg/L, 150 mg/L, 300 mg/L, and 500 mg/L extracted NAs, and 680 mg/L acetate (positive control).

Solutions with concentrations ten-fold higher than the desired concentration in the microcosms were prepared from the original 3100 mg/L NAs stock solution. Serum bottles received 2 mL substrate solution, 8 mL medium and 10 mL inoculum. A 5000 mg/L solution could not be prepared as it exceeded the concentration of the original extracted

NAs stock solution therefore a 2500 mg/L solution was prepared instead and 4 mL of this solution was added to the appropriate microcosms. August 1997 samples tested included MB1-5, MB1-15, MB1-20, MB2-5, MB2-15, MB2-20, DP-5, and MBIP1-5 and were incubated at 14°C. A second set of MB1-20 microcosms were incubated at room temperature. The amount of extracted NAs added to the microcosms was in excess of the *in situ* concentration of naphthenates in the inoculum.

2.5.3.3 Toxicity testing of the direct inhibition of methanogens by NAs

In order to determine whether NAs (naturally-occurring or commercially available) were directly inhibiting the methanogens present in the fine tailings, the following treatments were tested: no additions (negative control), 340 mg/L acetate (positive control), and acetate + 50 mg/L, 90 mg/L or 150 mg/L extracted NAs or commercial NAs (Kodak).

Serum bottles received 2 mL supplements, 8 mL medium, and 10 mL inoculum. As supplements, the control received 1 mL acetate solution (6800 mg/L) and 1 mL distilled water; the negative control received 2 mL distilled water; and each of the tests received 1 mL acetate solution and 1 mL NAs solution. August 1997 samples tested included MB1-5, MB1-20, MB2-5, MB2-20, DP-5, and MBIP1-5 which were incubated at room temperature. A second MB1-20 series was prepared and incubated at 14°C.

Two replicate sets of microcosms for sample MB1-20 were also prepared using H_2 instead of acetate as the additional substrate; one set was incubated at room temperature and the other at 14°C. The protocol was the same in preparing these microcosms, except that the 1 mL acetate solution additions were replaced with 1 mL water additions and 50 mL H_2 gas was added as an over pressure.

2.5.3.4 Potential for methane biogenesis from surrogate NAs

2.4.3.4.1 In sewage sludge

Four surrogate NAs plus one long chain fatty acid (hexadecanoic acid) were screened to determine if they could serve as carbon sources for methanogenesis (Figure

2.5). The surrogate NAs tested included 3-cyclohexylpropanoic acid, 4-cyclohexylbutanoic acid, and 5-cyclohexylpentanoic acid, all members of the Z= -2 family, and 6-phenylhexanoic acid. 6-Phenylhexanoic acid was chosen as a surrogate NA despite its aromatic character because it was the only chemically synthesized compound similar in structure to the other surrogate NAs that had a side chain greater than five carbons long. To determine whether the side chains of the surrogate NAs were susceptible to degradation, it was important to test as many different length carboxylic side chains as possible and since there was limited availability of the substituted cyclohexanes, it was necessary to use an aromatic compound. Each surrogate was tested at concentrations of 200, 400, 600, and 800 mg/L. The organic acids were insoluble in water but dissolved after the addition of a few drops of 10 M NaOH. Hexadecanoic acid was particularly difficult to dissolve, therefore it was prepared in heated 0.001 M NaOH and dispensed hot. Serum bottles (58-mL) received: 4 mL medium, 1 mL substrate solution and 5 mL inoculum. The medium containing hexadecanoic acid had a precipitate, and was inoculated with the assumption that increasing the total microcosm volume and consumption of the substrate by the microorganisms would shift the equilibrium and cause the precipitate to dissolve.

Microcosms containing 400 and 600 mg/L 3-cyclohexylpropanoic acid, 200 mg/L 4-cyclohexylbutanoic acid, and 200 and 400 mg/L 5-cyclohexylpentanoic acid were re-supplemented at 70 d with 1 mL of sterile ten-fold concentrated anoxic substrate solutions.

2.5.3.4.2 In sewage sludge and fine tailings

The addition of surrogate NAs to methanogenic microcosms was further studied using 3-cyclohexylpropanoic acid, 4-cyclohexylbutanoic acid, and 5-cyclohexylpentanoic acid and one long chain fatty acid (hexadecanoic acid). Each of these compounds was tested in concentrations of 200, 400 and 600 mg/L. Table 2.3 shows the predicted volumes of methane expected based on Buswell's equation. A 500 mg/L acetate positive control was prepared and should yield 5.2 mL CH_4, the same amount as the lowest projected amount of methane to be produced from the surrogate NAs (200 mg/L 3-cyclohexylpropanoic acid).

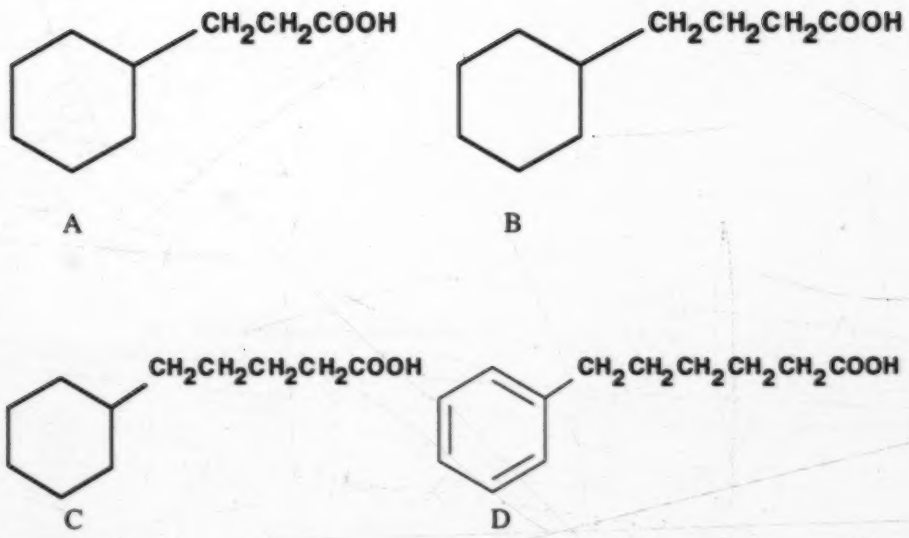


Figure 2.5: Chemical structures of the surrogate NAs studied. 3-cyclohexylpropanoic acid (A), 4-cyclohexylbutanoic acid (B), 5-cyclohexylpentanoic acid (C) and 6-phenylhexanoic acid (D).

Serum bottles received 1 mL H_2O (control) or 1 mL concentrated substrate stock solution (30-fold higher than the final target concentration in the microcosms), 4 mL anaerobic medium and 25 mL inoculum.

One set of microcosms was inoculated with sewage sludge obtained from the Gold Bar Wastewater Treatment Plant (Edmonton, AB) on July 29, 1998. Prior to use, the sewage sludge was stored at 22°C in a fume hood overnight to allow for some of the fermentable organic compounds to be consumed. Three other sets of microcosms were prepared using July 1998 fine tailings samples MBC1-8, MBC2-15 and MBWIP-10 as inocula. All microcosms were incubated at 22°C in the dark.

Microcosms containing fine tailings sample MBWIP-10 and 200 mg/L 3-cyclohexylpropanoic acid and 200 mg/L 4-cyclohexylbutanoic acid were re-supplemented after 228 d of incubation. Prior to re-supplementation, the total methane produced in each microcosm was measured and the total volume of dry methane generated was calculated (section 2.11.1). After this measurement, the microcosms were opened and flushed with O_2-free 30% CO_2 balance N_2 gas for at least 5 min to remove a majority of the methane from the microcosms. The bottles were then capped with new sterile serum stoppers and crimped shut. One milliliter of substrate stock solutions was added to the microcosms and the final concentration of 3-cyclohexylpropanoic acid or 4-cyclohexylbutanoic acid in the microcosms was ≈200 mg/L. The microcosms were shaken vigorously and allowed to equilibrate for at least 1 h, after which CH_4 was measured and found to be less than 0.16% vol CH_4 (lower detection limit of GC). The control microcosms were also flushed and crimped shut with new stoppers and the acetate-supplemented microcosms were re-supplemented. Methane levels in these microcosms plateaued after 113 d. Total gas produced was measured and the microcosms were again re-supplemented, however, the microcosms were not flushed to remove any produced methane.

Sewage microcosms were re-supplemented 271 d after inoculation, following the same protocol. Microcosms containing 400 and 600 mg/L 3-cyclohexylpropanoic and 200

mg/L 5-cyclohexylpentanoic acid were re-supplemented, along with the controls. After flushing and feeding, the microcosms contained less than 0.16 % vol CH_4 (lower detection limit of GC).

2.5.3.4.3 Sewage sludge enrichment

Sewage sludge was enriched for the degradation of 3-cyclohexylpropanoic acid (200, 400, 600 and 800 mg/L) and 5-cyclohexylpentanoic acid (200 mg/L). Serum bottles (158-mL) received 50 mL sewage sludge, 50 mL medium, 5 mL 20-fold substrate stock solution. The final volume of the microcosms was 106 mL, with a headspace of 52 mL. After 4 days of incubation, there was over 35% vol CH_4 in the headspace and so the microcosms were flushed for at least 10 min to return background levels to zero. After methane production was stimulated and plateaued, the total gas volume was measured. The bottles were opened and flushed, and after sealing were re-supplemented with fresh substrate. Each microcosm was re-supplemented twice. The volume of methane predicted by Buswell's equation is presented in Table 2.4.

2.5.3.5 Potential for anaerobic mineralization of a ^{14}C-bicyclic NA

A kind gift by Dr. M. Moore (Simon Fraser University, BC) enabled the establishment of microcosms to test the degradability of decahydro-2-naphthoic acid-8-^{14}C (DHNA, Figure 2.6), a bicyclic surrogate NA, to $^{14}CO_2$ and $^{14}CH_4$ under methanogenic conditions. Having the compound radiolabelled allows monitoring of the specific mineralization of this compound.

Serum bottles received 75 mL inoculum, 10 mL 7.5-fold concentrated medium and the radiolabelled chemical. Each microcosm received ≈0.5 µCi activity via a Whatman glass microfiber filter. The ^{14}C-DHNA had been previously dissolved in acetonitrile and had an activity of 5.0×10^4 dpm/µL. Therefore, 20 µL of this stock was applied to the glass filters and allowed to evaporate for at least 3 min. 2-^{14}C-Acetate was chosen as a positive control and ≈0.5 µCi activity was added to the microcosms. Sterile controls were prepared by

Table 2.3: Volumes of produced methane from the surrogate NAs predicted by Buswell's equation assuming complete mineralization of the whole compound in 30 mL microcosms containing fine tailings or sewage sludge.

Surrogate NA	Concentration in microcosms (mg/L)	Predicted CH_4 yield at STP (mL)
3-cyclohexylpropanoic acid	200	5.2
	400	10.3
	600	15.5
4-cyclohexylbutanoic acid	200	5.4
	400	10.7
	600	16.0
5-cyclohexylpentanoic acid	200	5.5
	400	11.0
	600	16.4
hexadecanoic acid	200	6.0
	400	12.1
	600	18.1

Table 2.4: Volumes of produced methane from the surrogate NAs predicted by Buswell's equation assuming complete mineralization of the whole compound in 100 mL microcosms containing sewage sludge.

Surrogate NA	Concentration in microcosms (mg/L)	Predicted CH_4 yield at STP (mL)
3-cyclohexylpropanoic acid	200	17.2
	400	34.5
	600	51.7
	800	68.8
5-cyclohexylpentanoic acid	200	18.3

autoclaving the inoculum for 1 h at 121°C for 3 consecutive days. Four replicates containing ^{14}C-DHNA and two replicates containing 2-^{14}C-acetate were prepared. Composite stoppers were used to seal microcosms containing July 1998 fine tailings samples, MBC2-5 or MBWIP-10. The production of $^{14}CO_2$ and $^{14}CH_4$ was monitored using the ^{14}C trapping system (section 2.9.9).

2.6 Study of the potential relationship between SRB and methanogens occurring within the fine tailings samples

To understand the relationship between the SRB and methanogens in the fine tailings consortium, an experiment was designed to establish whether the SRB were directly supplying methanogens with acetate. July 1998 fine tailings samples, MBC1-8 and MBC2-5, were used as inoculum. To each flushed 125-mL serum bottle 15 mL fine tailings inoculum, 8 mL bicarbonate medium, and 2 mL supplements were added (Table 2.5) for a final culture volume of 25 mL. Lactate was added to the appropriate microcosms to final concentration of 890 mg/L (10 mM). Sulfate (SO_4^{2-}) was added to some microcosms as a TEA supplement to a final concentration of 1000 mg/L. Molybdate (MoO_4^{2-}) was added as a selective SRB inhibitor to a final concentration of 660 mg/L (3 mM) as per Tanka and Lee (1997). Methane production and the concentrations of acetate, propionate, sulfate and lactate were monitored.

Two methanogen inhibitors were also used. The first, fluoromethane (CH_3F) (H&S Chemical Co. Inc., Covington, KY), an analogue of methane, was added at a concentration of 1 kPa to each microcosm (Janssen and Frenzel 1997). One-milliliter of CH_3F was injected into each microcosm (100-mL headspace) after inoculation. The microcosms were allowed to equilibrate and the concentration of CH_3F was measured using GC (section 2.9.8) and found to range between 0.85 and 0.97% vol corresponding to 0.85 and 0.97 kPa CH_3F, respectively. The second methanogen inhibitor tested was 2,4-pteridinediol (lumazine) at a concentration of ≈100 mg/L (0.6 mM) according to Nagar-Anthal et al. (1996).

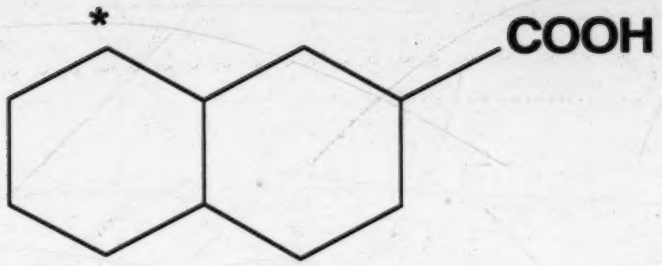


Figure 2.6: Chemical structure of decahydro-2-naphthoic acid (DHNA). * denotes position of ^{14}C.

Table 2.5: Summary of the different treatment microcosms used to study the relationship between SRB and methanogens and the additions that each received.

Treatment	Lactate (mL)[a]	SO_4^{2-} (mL)[b]	CH_3F (mL)	Lumazine (mL)[c]	MoO_4^{2-} (mL)[d]	H_2O (mL)
Control	0	0	0	0	0	2
Lactate	1	0	0	0	0	1
Lactate + CH_3F	1	0	1	0	0	1
Lactate + CH_3F + SO_4^{2-}	1	.5	1	0	0	.5
Lactate + MoO_4^{2-}	1	0	0	0	.5	.5
Lactate + MoO_4^{2-} + SO_4^{2-}	1	.5	0	0	.5	0
Lactate + SO_4^{2-}	1	.5	0	0	0	.5
Lactate + Lumazine	1	0	0	.5	0	.5
Lactate + SO_4^{2-} + Lumazine	1	.5	0	.5	0	0
Control + Lumazine[e]	0	0	0	.5	0	1.5
Control + CH_3F[e]	0	0	1	0	0	2

[a]lactate stock concentration was 22 g/L
[b]sulfate stock concentration was 50 g/L
[c]lumazine stock solution was 4.9 g/L
[d]molybdate stock solution was 33 g/L
[e]treatments only tested on fine tailings sample MBC1-8

2.7 Effects of prolonged sample storage on the fine tailings

Four fine tailings samples collected in July 1998 (MBC1-5, MBC2-5, MBC2-15 and MBWIP-10) were stored in the dark at 4°C, 14°C and room temperature (22°C). Subsamples (750 mL) were removed from the original 4-L sampling bottles under a constant flow of O_2-free 30% CO_2 balance N_2 gas and dispensed into sterile preflushed 1-L amber bottles. The amber bottles were placed at the appropriate storage temperatures. The samples incubated at 4°C were left in the original sampling bottles.

Three parameters were studied over time: the response of the fine tailings samples to acetate supplementation and the change in methanogen and SRB population sizes (MPN values). The acetate-supplementation experiments involved microcosms with 9 mL medium, 20 mL inoculum and 1 mL of H_2O (control) or 1 mL 30 g/L acetate (treatments) resulting in a final concentration of 1000 mg/L acetate. Samples stored at 4°C and 14°C were enumerated for acetate-utilizing methanogens and SRB using the standard 5-tube MPN method at each time. H_2-utilizing methanogens were enumerated less frequently (0, 3, and 9 mon). MPN enumeration of the samples stored at room temperature occurred after 9 mon storage only.

The inoculation schedule followed was:

- Time 0: immediately after collection (July 16, 1998)
- Time 1: after 1 mon storage (August 14, 1998)
- Time 3: after 3 mon storage (October 15, 1998)
- Time 6: after 6 mon storage (January 13, 1999)
- Time 9: after 9 mon storage (April 20, 1999)

2.8 Estimating ultimate methane production from fine tailings

2.8.1 August 1997 samples: 250-mL microcosms

To determine the methane yield of the fine tailings, microcosms were incubated over a long term (18 to 20 mon) and the volume of methane produced was measured over time. Microcosms containing 250 mL inoculum were prepared in 500-mL glass reagent

bottles under anaerobic conditions and each microcosm received 1.0 mg/L resazurin. The bottles were sealed with a sterile rubber stopper held in place with a specially designed plastic clamp to create a gas-tight system. Three August 1997 samples were tested; MB1-5, MB2-5 and MBIP1-5. The samples had been stored at 4°C for 6 mon when the microcosms were established. Two microcosms for each sample were prepared with one incubated at room temperature and the other at 14°C. Gas production was measured using the apparatus and method described by Fedorak and Hrudey (1983) (Appendix B). Prior to gas measurements, the microcosms incubated at 14°C were allowed to reach room temperature to minimize any effects a reduced temperature would have on the gas pressure in the microcosms, which would influence the gas measurement taken.

2.8.2 August 1997 samples: 100-mL microcosms

Three months after the 250-mL microcosms had been established, 100-mL microcosms were prepared in 158-mL serum bottles. These microcosms were set up following the same protocol as the 250-mL microcosms. Quadruple replicates were prepared, three of which were analyzed on a regular basis for 17 mon. At the time of inoculation, MB1-5, MB2-5 MBIP1-5 and DP-5 had been stored for 9 mon at 4°C.

2.8.3 July 1998 samples: 100-mL microcosms

The methane yield of five July 1998 samples was monitored including MBC1-5, MBC1-8, MBC2-5, MBC2-15, and MBWIP-10. For each sample, ten 100-mL microcosms were prepared: five were incubated at room temperature and five at 14°C. Three replicates of each set of microcosms were analyzed regularly for over 15 mon.

2.9 Analytical methods

2.9.1 Analysis of methane production

Methane concentration (% vol) was analyzed using a Hewlett Packard 5700A GC with a flame ionization detector fitted with a 2 m x 0.3 cm column packed with Tenax GC (60/80 mesh). N_2 was the carrier gas at 50 mL/min, and H_2 and air had flow rates of 35

mL/min and 300 mL/min, respectively. The injector, oven and detector temperatures were 40°C, 40°C, and 250°C, respectively. Under these conditions, methane had a retention time of ≈0.23 min. Microcosms were shaken and 0.1 mL of headspace gas was removed for analysis. Chromatograms and peak areas were obtained using a HP 3380A integrator.

For some microcosms, a total gas measurement was determined using the gas volume measuring apparatus described in Appendix B. The total volume of methane at STP was calculated from the total volume of gas produced and the methane (% vol) measurements (as described in section 2.11.2). Measuring total gas volume was a more involved analysis than measuring methane (% vol) via GC. Over 1700 microcosms were monitored throughout the project, therefore methane (% vol) was measured on a regular basis as gas volume measurements would have been too time-consuming. Gas volume measurements were reserved for situations in which the total volume of gas produced was needed (section 2.8) or if there was a comparison made between produced and expected amount of methane.

2.9.2 Carbon dioxide production and methane utilization

Carbon dioxide production and methane utilization by methanotrophs in the MPN determinations were analyzed using a Varian gas chromatograph (Model 700) with a thermal conductivity detector fitted with a 3 m x 0.3 cm column packed with Poropak R (60/80 mesh) with a carrier gas of helium. The column temperature was 140°C and the injector and detector temperatures were 100°C. Each MPN tube was mixed and 0.1 mL or 0.5 mL of headspace was removed for analysis. Retention times for methane and carbon dioxide were 1.30 and 2.20 min, respectively. Chromatograms and peak areas were obtained using a HP 3380A integrator.

2.9.3 Analysis of phenol and cresols

To monitor the concentration of phenolic compounds in methanogenic microcosms, subsamples were collected and analyzed. The microcosms were shaken, and 0.05-mL

liquid subsamples were removed and injected into 0.6-mL microcentrifuge tubes. The subsamples could then be analyzed immediately or stored at -20°C for later analysis. At the time of analysis, the subsamples were spun in a microcentrifuge for 10 min at 14000 rpm in order to pellet the solids. After centrifugation, a 2-µL supernatant sample was removed using a 10-µL glass Hamilton syringe and injected into a GC for quantification.

The concentration of phenol and the three cresol isomers was monitored with a Hewlett Packard 5790A GC with a flame ionization detector fitted with a 2 m x 0.32 cm (outer diameter) stainless steel column packed with a 60/80 mesh modified coated Tenax TA (formerly Tenax GC, Supelco 1-1982) (Bartle and Elstub 1977). The flow rates were 51 mL/min N_2 (carrier gas), 350 mL/min air and 45 mL/min H_2. The oven, injector and detector temperatures were set at 190°C, 250°C, and 350°C, respectively. Calibration curves for each of the compounds were generated using solution concentrated up to 300 mg/L (Appendix C). Once reliable calibration curves had been produced, each time the instrument was used only the 100 mg/L standard was used to calibrate the GC. Chromatograms and peak areas were obtained using a HP 3380A integrator.

2.9.4 Measurement of sulfate

Sulfate measurements were performed by Brian Rolseth in the Limnology Laboratory, Department of Biological Sciences or by Myrna Salloum in the Department of Renewable Resources, both at the University of Alberta. Samples were prepared for analysis by subsampling the microcosms with a sterile 1-mL syringe fitted with an 18-G 1.5 inch needle. The samples were spun down in a microcentrifuge for 10 min. The supernatant was then filter sterilized through a 0.22 µm pore size filter into sterile Eppendorf tubes. The samples were then stored in the refrigerator until analyzed. Prior to analysis, dilutions of the filter-sterilized samples were prepared with Milli-Q water to have a final sample volume between 4 and 5 mL within the concentration range of 2.0 to 20 mg/L. Sulfate was separated from the other ions in the sample through ion chromatography

and then detected and quantified with a conductivity detector. The Limnology Laboratory used a Dionex model 2000i ion chromatograph with a IonPac AS4A-SC column (Dionex Canada Ltd., Oakville, ON). The mobile phase was 1.7 mM sodium bicarbonate and 1.8 mM sodium carbonate. The Department of Renewable Resources used a Dionex model 4000i ion chromatograph fitted with a Dionex IonPac IC column. The mobile phase was 1.0 mM sodium bicarbonate and 3.5 mM sodium carbonate. A standard curve was run with each analysis (Appendix D). Data were collected using Dionex Peaknet 4.30 software.

2.9.5 Measurement of acetate and propionate

Acetate and propionate measurements were performed on a Varian 3600 GC equipped with a flame ionization detector and a DB-FFAP capillary column of 15 m length with an internal diameter of 0.53 mm obtained from Chromatographic Specialties (Brockville, ON). Helium was the carrier gas at 30 mL/min and N_2 was the make-up gas at 20 mL/min. H_2 and air had flow rates of 30 and 300 mL/min, respectively. The temperature program started with a column temperature of 120°C which was held for 1 min. The temperature was then increased at a rate of 8°C/min to a final temperature of 130°C. The retention time of acetate and propionate were ≈0.67 and ≈0.87 min, respectively.

Samples (1 mL) were clarified in a microfuge for 10 min and 50 µL supernatant were transferred to a clean Eppendorf to which 10 µL of 4 M H_3PO_4 were added. Two microliters of the sample were then injected into the GC for analysis. Calibration curves were run each time a set of samples were analyzed (Appendix E). The GC had linear sensitivity to acetate and propionate over the range of 0 to 1000 mg/L. Samples which had concentrations of acetate greater than 1000 mg/L were diluted. Chromatograms and peak areas were obtained using Varian Star Workstation software.

2.9.6 Measurement of lactate

Lactate measurements were performed on a Hewlett Packard 5890 GC equipped with a flame ionization detector and a DB-5 capillary column of 30 m length with an

internal diameter of 0.25 mm obtained from Chromatographic Specialties. Helium (carrier gas), H_2 and air had flow rates of 29, 30 and 300 mL/min, respectively. The temperature program began with a column temperature of 60°C which increased to 100°C at a rate of 10°C/min and held for 1 min. The injector and detector were both set at 250°C.

Supernatant samples (100 to 150 µL) underwent a pre-extraction step with 200 µL chloroform to remove some of the interfering organic compounds present. Pre-extraction was performed in 1-mL glass Reacti-vials (Pierce Chemical Company, Rockford, IL). The vials were capped with Teflon-lined caps, inverted 10 to 20 times and the contents briefly microfuged. The aqueous sample (100 µL) was transferred to a 4-mL standard glass vial for derivatization. To 100 µL of the aqueous portion, 100 µL of methanol containing 4 mg/mL malonic acid as an internal standard was added, plus 50 µL 4 M H_3PO_4. The vial was sealed with a Teflon lined cap and heated for 30 min at 60°C. After heating, the sample was transferred into a clean glass Reacti-vial and 100 µL water and 50 µL chloroform were added to extract the methylated derivative. The vial was capped and inverted 20 times and microfuged briefly. Two microliters of the chloroform layer were injected into the GC. The lactate derivative had a retention time of ≈2.74 min and the malonic acid derivative eluted at ≈4.41 min. The GC response was linear for lactate up to 1000 mg/L (Appendix F).

2.9.7 Measurement of nitrous oxide

The presence of N_2O in microcosm headspace was analyzed using a Hewlett Packard 5890 series II GC with a thermal conductivity detector fitted with a 2 m x 0.3 cm column packed Poropak R (60/80 mesh). Helium was the carrier gas at 59 mL/min. The injector, oven and detector temperatures were set at 37°C, 35°C, and 80°C, respectively. Headspace (0.1 mL) was removed for analysis. The retention time of N_2O was ≈2.75 min.

2.9.7 Measurement of CH_3F

CH_3F was measured using the same Hewlett Packard 5700A GC used for methane analysis. Headspace samples (0.1 mL) were removed from the microcosms and injected

into the GC. CH_3F has a retention time approximately twice as long as methane, therefore there is no interference by any methane present in the sample. Analysis of standards indicated that the GC had a linear response up to 4% vol CH_3F, which is consistent with previous reports (Frenzel and Bosse 1996).

2.9.9 Quantification of $^{14}CO_2$ and $^{14}CH_4$

The potential mineralization of ^{14}C-labelled compounds required the ability to monitor for production of $^{14}CO_2$ and $^{14}CH_4$. Using an oxidation oven from a Packard model 894 gas proportional counter (GPC), the trapping systems described by Nuck and Federle (1996) and Fedorak et al. (1982) were combined in order to detect $^{14}CO_2$ and $^{14}CH_4$ (Figure 2.7). The trapping system contained two sets of trapping vials. One set of two scintillation vials was isolated from the oxidation oven (vials 1A and 1B) and the second set was positioned after the oxidation oven (vials 2A and 2B). The system was designed so that 1 mL of headspace gas would be injected into a helium flow and pass to vials 1A and 1B to trap $^{14}CO_2$. A slight modification to the system diverted the helium flow away from trap 1, and through the oxidation oven to vials 2A and 2B. A second 1 mL headspace sample was then injected into the helium flow and the sample would by-pass the first set of traps and travel through the oxidation oven (800°C) where $^{14}CH_4$ would be oxidized to $^{14}CO_2$ and trapped in vials 2A and 2B. The amount of $^{14}CH_4$ produced would then be calculated by subtracting the radioactivity in vials 1A and 1B from the radioactivity in vials 2A and 2B.

Helium as a carrier gas was connected to a flow controller set at 40 mL/min. An injection port was created at an intersection in the GPC gas lines with a plastic "T". One arm of the "T" connected into the gas line, the second arm became the injection port when covered with a small serum stopper, and Tygon tubing was connected to the third arm to carry helium to the first trap.

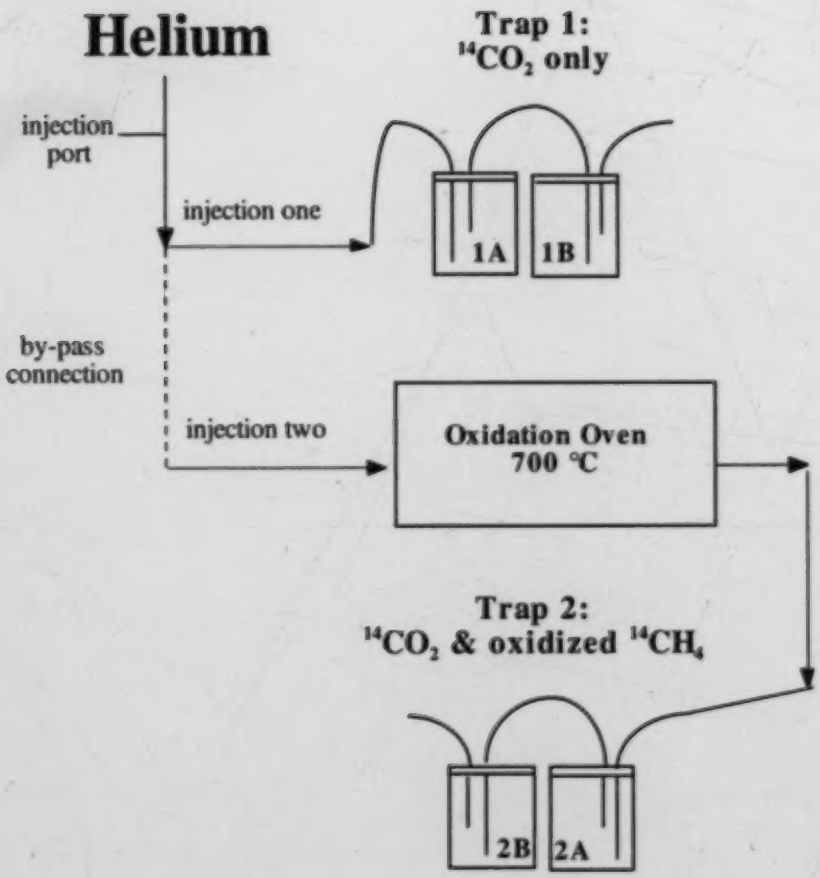


Figure 2.7: Schematic diagram of the trapping system used to detect $^{14}CO_2$ and $^{14}CH_4$.

Each two-vial trap was a specially designed flushing apparatus. Scintillation vial caps were modified so that two blunted 18-G stainless steel needles were soldered onto a piece of metal. Holes had been bored into the vial cap and a rubber liner was sandwiched between the metal plate and the underside of the cap so the needles protruded through into the scintillation vial. The three layers were secured together with a small screw and silicone sealant to prevent gas leaks. One of the blunted 18-G needles was 1.25 inches long so that when the vial contained 11 mL of fluor and trapping agent, it was submerged to near the bottom of the vial while the other needle (0.50 inches long) was above the level of the fluor. Externally, Tygon tubing was used to connect the "short" 18-G needle of first vial to the "long" 18-G needle of the second vial. With this design, gas would flow directly into the fluor of the first vial where the $^{14}CO_2$ would be trapped, any non-trapped (excess) $^{14}CO_2$ would partition into the headspace and be forced into the second vial and be trapped. Each vial contained 10 mL of the scintillation cocktail ACS fluor (Amersham Canada Ltd., Oakville, ON) and 1 mL Carbosorb (Packard, Meriden, CT) to trap $^{14}CO_2$.

Prior to use and on an ongoing basis, the system was thoroughly monitored for gas leaks, and He flow rates were measured at various points in the system. The trapping efficiency of the two sets of trapping vials was confirmed to be essentially 100% using $H^{14}CO_3$, and the ability of the trapping system to trap and quantify the production of $^{14}CH_4$ was established using ^{14}C-glucose and ^{14}C-acetate (Appendix G.5, Table G1). Generally the first vial in each set (1A and 2A) trapped 100% of the activity in the headspace sample therefore, for future analysis, only the 1A and 2A vials were replaced between replicates and the 1B and 2B vials were left to pool any activity potentially missed by the first vials.

After injection of the headspace sample, the system was allowed to flush for at least 3 min. After flushing, the vials were capped and wiped with a KimWipe to remove any outside contamination. The vials were placed in the scintillation counter and allowed to dark adapt overnight. After dark adaptation, the refrigeration unit of the scintillation counter was

turned on and the samples were then analyzed. Activity counts were normalized against background levels.

The development of a functional system was the result of many preliminary attempts and improvements which led to the determination of optimal operating conditions. Preliminary trials and results are documented in Appendix G.

2.9.10 Pore water analysis done by Syncrude Canada Ltd.

During sampling at the MLSB, subsamples were collected for subsequent pore water analysis by Syncrude Canada Ltd. personnel. A variety of chemical and physical parameters were evaluated with methods used by Syncrude Canada Ltd. (Syncrude 1995). Major anions (Cl^-, SO_4^{2-}) and NH_3 were separated by ion chromatography with a Dionex-DX 300 series ion chromatograph fitted with an IonPac AG4A-SC column with a sodium bicarbonate (3 mM) and sodium carbonate (2.4 mM) mobile phase and detected with a conductivity detector (Syncrude 1995). Cations (Mg^{2+}, Ca^{2+}, Na^+ and K^+) were analyzed by an Inductively Coupled Argon Plasma Atomic Emission Spectrometer model 3580 (Applied Research Laboratories, Valencia, CA). Bicarbonate (HCO_3^-) concentrations were either determined through titration (alkalinity) or by calculating the value from dissolved inorganic carbon values. NAs concentrations were determined with a FT-IR spectroscopy method (Jivraj et al. 1995). Naphtha content was determined by solvent extraction and GC analysis on a Hewlet Packard 5890 GC fitted with a flame ionization detector and two columns with complementary polarity (Syncrude 1995). Bitumen and solids content were determined concurrently. Solvent extraction isolated the bitumen which was then dispensed onto filter paper and quantified gravimetrically and the solids were collected and dried to a constant weight (Syncrude 1995). Toxicity data were obtained using the standard Microtox assay (Herman et al. 1994).

2.10 Statistical methods

2.10.1 Cochran's method for testing MPN values

The most probable number of organisms (MPN) was calculated from the combination of positive results using a standard 5-tube MPN statistical table (Standard Methods 1985). The 95% confidence intervals for the MPN values were also provided by the 5-tube MPN statistical table. Cochran's statistical method (Cochran 1950), equation [2.1], was used to compare MPN values obtained from 5-tube MPN method using 10-fold serial dilutions. The critical value for Z at $P<0.05$ is 1.96, therefore if the calculated value of Z was greater than 1.96, then the two MPN values were considered statistically different. The "critical ratio" of MPN_1/MPN_2 was determined to be 5.2, as shown below.

$$Z = \frac{|\log MPN_1 - \log MPN_2|}{(0.58)\,(1/5 + 1/5)^{1/2}} \qquad \text{equation [2.1]}$$

$$1.96 = \frac{|\log MPN_1 - \log MPN_2|}{0.3668}$$

$$1.96 \times 0.3668 = |\log MPN_1 - \log MPN_2|$$

$$0.719 = \log \frac{|MPN_1|}{|MPN_2|}$$

$$10^{0.719} = \frac{|MPN_1|}{|MPN_2|} = 5.2$$

Therefore, if $MPN_1/MPN_2 \geq 5.2$, then the MPN values were considered significantly different at $P<0.05$.

2.10.2 Dunnett's method for methane production .

Methane values (% vol) were analyzed using the method of Dunnett (1955) to determine which treatments produced methane levels significantly different from the control values ($P<0.05$). For clarity, the figures presented throughout this thesis show only the standard deviations of the mean methane production in the control microcosms, rather than for all microcosms. Statistically significant differences between methane concentrations in the treatment microcosms and the controls are discussed within the text.

2.11 Calculations

2.11.1 Buswell's equation for estimating theoretical methane production

Using a equation [2.2] derived by Buswell and Muller (1952), one can calculate the expected theoretical amount of methane to be produced by the addition of a given substrate consisting of carbon, hydrogen and oxygen. Buswell's equations for the potential methanogenic substrates tested are presented in Table 2.6.

$$C_nH_aO_b + [n - \frac{a}{4} - \frac{b}{2}]\ H_2O \text{ ----------> } [\frac{n}{2} - \frac{a}{8} + \frac{b}{4}]\ CO_2 + [\frac{n}{2} + \frac{a}{8} - \frac{b}{4}]\ CH_4 \quad \text{equation [2.2]}$$

Knowing how many moles of a given substrate are added to a microcosm, the expected number of moles of methane can be calculated which can be converted to the volume of methane expected as 1 mol CH_4 = 22.4 L CH_4 (at STP). The expected volume of methane can then be converted to the expected CH_4 (% vol) to be produced according to the equation [2.3].

$$CH_4\ (\%\ vol) = \frac{[a]}{[a+b]} \times 100 \quad \text{equation [2.3]}$$

where:

a = predicted volume (mL) of methane to be produced

b = volume (mL) of headspace in microcosm

2.11.2 Conversion of CH_4 (% vol) values to volume (mL) CH_4 produced

Comparing CH_4 (% vol) values is convenient, however in some situations the volume of CH_4 produced was compared to Buswell's predicted values. Total gas measurements were taken using the apparatus described in Appendix B. Briefly, excess headspace gas caused the displacement of the syringes of the gas measuring apparatus as pressure equilibrated to atmospheric pressure. The total volume of excess gas produced in the microcosm was indicated by the amount of gas contained in the syringes. The total volume of gas, and the methane concentration (% vol) were used to convert methane values from a "wet" % vol value to a "dry" volume at STP using equation [2.4].

Table 2.6: Buswell's equations for the compounds tested as potential methanogenic substrates.

Compound	Buswell's Equation
acetic acid (acetate)	$CH_3COOH + 0.25\ H_2O \Rightarrow 1.125\ CO_2 + 0.875\ CH_4$
phenol	$C_6H_6O + 4.00\ H_2O \Rightarrow 2.50\ CO_2 + 3.50\ CH_4$
cresols (*m*-, *p*-, *o*-)	$C_7H_8O + 4.50\ H_2O \Rightarrow 2.75\ CO_2 + 4.25\ CH_4$
3-cyclohexylpropanoic acid	$C_9H_{16}O_2 + 4.00\ H_2O \Rightarrow 3.00\ CO_2 + 6.00\ CH_4$
4-cyclohexylbutanoic acid	$C_{10}H_{18}O_2 + 4.50\ H_2O \Rightarrow 3.25\ CO_2 + 6.75\ CH_4$
5-cyclohexylpentanoic acid	$C_{11}H_{20}O_2 + 5.00\ H_2O \Rightarrow 3.50\ CO_2 + 7.50\ CH_4$
6-phenylhexanoic acid	$C_{12}H_{16}O_2 + 7.00\ H_2O \Rightarrow 4.50\ CO_2 + 7.50\ CH_4$
hexadecanoic acid	$C_{16}H_{32}O_2 + 7.00\ H_2O \Rightarrow 4.50\ CO_2 + 11.5\ CH_4$

$$\text{Dry volume of } CH_4 \text{ at STP (mL)} = (S+V) \times \frac{(P-P_w)}{(T+273)} \times \frac{CH_4}{c.f.} \times \frac{273^\circ K}{760 \text{ mm Hg}} \qquad \text{equation [2.4]}$$

where:

S = total volume (mL) of gas contained in the syringes

V = headspace volume (mL) in microcosms

P = barometric pressure (mm Hg) adjusted to STP which includes correction for temperature, gravity and elevation (PRINCO 1983)

P_w = water vapor pressure (mm Hg) at T

T = temperature (°C)

CH_4 = % vol methane in the headspace as determined by GC analysis

c.f. = correction factor for water saturation which can be calculated by the formula: 1-[(D x 22.4 L/mole)/(18 g/mole x 1000)] where D is the density of water vapor at saturation (g/m^3) (List 1958).

3. Results and Discussion

3.1 Chemical, physical and toxicity properties of fine tailings samples

The temperature, pH, dissolved organic carbon (DOC) and oil and solids content of the fine tailings samples were determined (Tables 3.1 and 3.2). The temperature of the fine tailings samples was measured at the time of collection. At the surface (1 m samples), the temperature was close to ambient ≈20°C. The fine tailings samples had temperatures below ambient, dropping as low as 11°C. The temperature of the fine tailings samples does not fluctuate significantly and generally averages between 11 and 15°C year round (M. MacKinnon, personal communication). The pH of the samples was relatively constant ranging between 8.1 and 8.5, which is consistent with reported values (FTFC 1995a). The solids content of the fine tailings samples increased with depth, resulting from the settling and consolidating process of the fine tailings within MLSB. The oil content of the samples ranged between 0.5 and 3.0g/100g which are normal levels of unrecovered oil in the fine tailings (FTFC 1995a). DOC values ranged between 43 and 57 mg/L.

Pore waters obtained from the fine tailings samples were analyzed for major cations and anions and toxicity (Tables 3.1 and 3.2). The concentrations of sodium (Na^+) (Figure 3.1), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}) and ammonia (NH_3) changed little with depth of sample or between the two sampling periods. Chloride (Cl^-) ions were high in the water samples and dropped by half in the fine tailings samples, but remained at the same concentration (200 to 300 mg/L) regardless of sample depth. The concentration of bicarbonate (HCO_3^-) was over 1000 mg/L for all of the samples and remained constant throughout the profile of samples collected (Figure 3.1), attesting to the high buffering capacity of the tailings pond. The most drastic change in ion concentration was sulfate (SO_4^{2-}). Concentrations of sulfate were highest in the surface waters and then dropped drastically as the depth of the sample increased (Figure 3.1). The most significant drop in sulfate levels was noted in samples collected in 1997 (Figure 3.1A and B); below 5 m there was little sulfate present.

Table 3.1: Properties of fine tailings samples collected from MLSB, Base Mine Lake and Demonstration Pond in August 1997. Data were provided by M. MacKinnon of Syncrude Canada Ltd.

Parameter	MB1-1	MB1-5	MB1-10	MB1-15	MB1-20	MB2-1	MB2-5	MB2-10	MB2-15	MB2-20
Depth (m)	1	5	10	15	20	1	5	10	15	20
pH	8.1	—[a]	—	—	—	8.1	—	—	—	—
Temp (°C)	20.2	17.7	17.8	21.2	20.6	20.2	16.5	15.3	16.3	15.7
Solids (g/100g)	—	19.9	33.2	39.0	39.8	—	24.7	31.5	39.1	36.1
Na^+ (mg/L)	782	533	624	627	656	775	593	488	551	512
K^+ (mg/L)	9.1	10.3	12.6	12.5	12.0	9.3	10.5	10.1	11.1	10.7
Mg^{2+} (mg/L)	4.3	2.6	5.0	4.4	4.1	4.0	2.7	3.7	5.1	4.4
Ca^{2+} (mg/L)	5.9	3.4	5.8	5.3	5.1	5.8	4.0	5.2	7.2	6.4
Cl^- (mg/L)	536	195	265	284	298	531	260	129	138	126
SO_4^{2-} (mg/L)	146	4.9	2	3.3	3.9	146	4.1	4.9	2.8	2.8
HCO_3^- (mg/L)	960	1070	1240	1250	1020	950	1130	1100	1280	1200
NH_3 (mg/L)	6.7	8.8	7.6	7.5	7.5	6.8	8.0	7.8	7.7	9.0
NAs (mg/L)	84	63	65	—	88	—	66	65	—	57
DOC (mg/L)	52	48	46	51	43	51	49	49	45	47
Oil (g/100g)	—	0.9	1.3	1.3	1.5	—	1.7	1.6	2.6	2.4
Naphtha (g/100g)	<.01	0.01	0.02	0.01	0.02	<.01	0.02	0.07	0.06	0.06
Microtox IC_{50} (%)	29	—	55	—	20	55	—	20	—	—

[a]parameter was not measured on this sample

Table 3.1 continued

Parameter	DP-1	DP-5	MBIP1-1	MBIP1-10
Depth (m)	1	5	1	10
pH	8.6	—	8.0	—
Temp (°C)	17.0	15.9	20.0	15.9
Solids (g/100g)	<20	31.2	0.03	25.2
Na^+ (mg/L)	149	491	727	749
K^+ (mg/L)	3.5	11.0	9.3	23.1
Mg^{2+} (mg/L)	15	3.6	6.0	9.2
Ca^{2+} (mg/L)	25	5.9	8.5	8.1
Cl^- (mg/L)	39	166	388	362
SO_4^{2-} (mg/L)	94	9	53	3.2
HCO_3^- (mg/L)	330	950	960	1410
NH_3 (mg/L)	0.01	4.3	8.8	7.9
NAs (mg/L)	6.5	72	—	68
DOC (mg/L)	32	54	48	57
Oil (g/100g)	—	0.93	<0.1	1.6
Naphtha (g/100g)	—	0.01	<0.01	.14
Microtox IC_{50} (%)	100	32	50	38

Table 3.2: Properties of fine tailings samples collected from MLSB, Base Mine Lake and Demonstration Pond in July 1998. Data were provided by M. MacKinnon of Syncrude Canada Ltd. Due to differences in sample designations between Syncrude Canada Ltd. personnel and F. Holowenko, the values reported correspond to either MBC1 or MBC2 samples, but it is impossible to determine which MBC samples correspond to sample MBC1 or MBC2.

Parameter	MBC -1	MBC-5	MBC-8	MBC-10	MBC -15	MBC -20
Depth (m)	1	5	8	10	15	20
pH	8.3	8.3	—[a]	8.3	8.5	8.5
Temp (°C)	22.0	14.0	13.5	12.5	11.0	11.5
Solids (g/100g)	—	15.5	22.4	25.0	38.8	47.7
Na^+ (mg/L)	895	590	667	541	438	444
K^+ (mg/L)	12.9	15	18.5	14.6	11.9	12.2
Mg^{2+} (mg/L)	9.7	7.3	6.6	8.0	6.9	5.7
Ca^{2+} (mg/L)	7.3	6.1	5.2	6.0	4.6	4.4
Cl^- (mg/L)	634	386	307	301	150	127
SO_4^{2-} (mg/L)	173	87	68	68	17	7
HCO_3^- (mg/L)	1020	1010	1030	970	1000	1080
NH_3 (mg/L)	9.0	10	10	10	9.3	8.7
NAs (mg/L)	86	61	—	79	66	63
DOC (mg/L)	—	—	—	—	—	—
Oil (g/100g)	—	0.62	0.93	1.8	3.1	2.6
Naphtha (g/100g)	—	0.04	0.02	0.07	0.11	0.10
Microtox IC_{50} (%)	30	39	—	27	16	17

[a]parameter was not measured on this sample

Table 3.2 continued

Parameter	98DP-1	98DP-5	MBWIP-1	MBWIP-10
Depth (m)	1	5	1	10
pH	8.5	8.3	8.2	8.3
Temp (°C)	20.5	17.5	22.0	12.5
Solids (g/100g)	—	30.8	0.03	32.3
Na^+ (mg/L)	173	513	734	780
K^+ (mg/L)	3.8	18.3	17.0	25.3
Mg^{2+} (mg/L)	16	4.3	6.4	6.8
Ca^{2+} (mg/L)	23	4.2	8.2	9.3
Cl^- (mg/L)	43	158	390	370
SO_4^{2-} (mg/L)	100	6.2	26	2
HCO_3^- (mg/L)	370	900	1330	1530
NH_3 (mg/L)	0.2	5.9	4.1	7.3
NAs (mg/L)	8.3	72	61	66
DOC (mg/L)	30	52	—	—
Oil (g/100g)	—	1.0	—	1.9
Naphtha (g/100g)	—	0	—	0.10
Microtox IC_{50} (%)	100	24	25	37

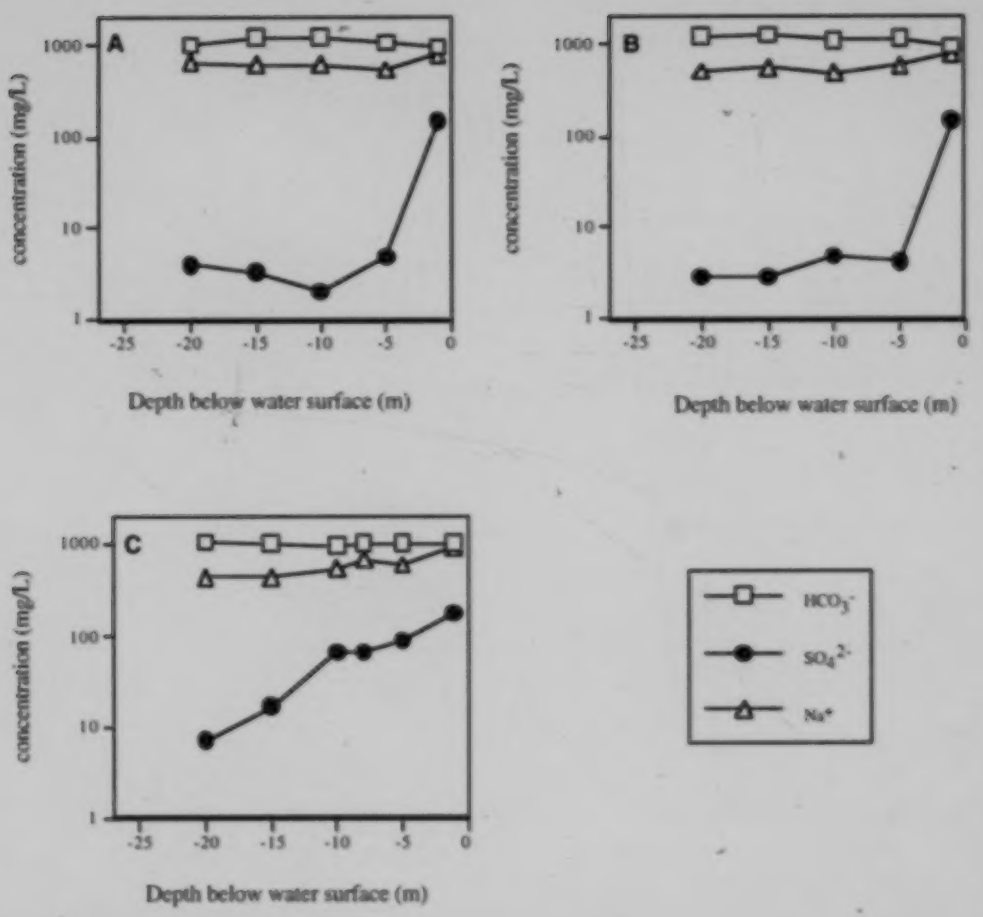


Figure 3.1: Concentration of bicarbonate (HCO_3^-), sulfate (SO_4^{2-}) and sodium (Na^+) ions as the depth of the fine tailings increased below the surface of the water in the MLSB in August 1997 sites 1 (A) and 2 (B) and July 1998 (C).

The concentrations of NAs were variable (Tables 3.1 and 3.2) but within normal parameters (FTFC 1995a), ranging between 60 and 85 mg/L. NAs have been linked with the toxicity of the pore waters and comparison of the NAs concentrations and the Microtox IC_{50} values showed correlation between the two. Decreasing IC_{50} values relate to higher toxicity, samples with low IC_{50} values had higher NAs concentrations. For example MB1-5 had a NAs concentration of 65 mg/L and an IC_{50} value of 55% whereas MB1-1 had a NA concentration of 84 mg/L and an IC_{50} value of 29% (Table 3.1).

The above mentioned trends were consistent for all of the samples collected in 1997 and 1998 from the MLSB and the Base Mine Lake. The exception were the samples collected from the Demonstration Pond, particularly the water samples (1 m). DOC values in the water were at a lower level (30 mg/L) as were the concentrations of Na^+, Cl^-, SO_4^{2-} and HCO_3^-. Conversely, Ca^{2+} and Mg^{2+} concentrations were higher in the Demonstration Pond waters than the MLSB water samples. NAs were depleted with concentrations in the Demonstration Pond water ranging between 6.5 and 8.3 mg/L, resulting in low toxicity (as indicated by the high IC_{50} values). Demonstration Pond is a wetland whereas the MLSB and the Base Mine Lake are settling ponds, it is not surprising then that the chemistry of the Demonstration Pond water is vastly different from the others.

3.2 Enumeration of microorganisms

3.2.1 Evaluation of the MPN assay with fine tailings samples

Because the 5-tube MPN assay was the only method used to enumerate the populations of microorganisms in the fine tailings samples, it was important to establish the reliability and reproducibility of the MPN values obtained. To do this, duplicate MPN assays were prepared with July 1998 samples MBC1-5 and MBWIP-10 using two complete dilution series prepared for each sample.

MBC1-5A and MBC1-5B had statistically similar SRB and acetate-utilizing methanogen MPN values (10^4/g) but slightly different H_2-utilizing methanogen MPN values (Table 3.3) as indicated by Cochran's statistical method and by examination of the

Table 3.3: Enumeration results of duplicate MPN analysis on fine tailings samples. Values are expressed as MPN/g dry weight. The critical MPN_1/MPN_2 ratio for statistical significance was 5.2.

	MBC1-5		MBWIP-10	
Population	**Replicate A**	**Replicate B**	**Replicate A**	**Replicate B**
H_2-utilizing methanogens	1.9×10^6 (7.7×10^5 to 6.5×10^6)[b]	1.2×10^5 (3.9×10^4 to 4.6×10^5)	2.2×10^7 (8.7×10^6 to 6.7×10^7)	1.0×10^7 (3.9×10^6 to 3.8×10^7)
acetate-utilizing methanogens	1.9×10^5 (7.7×10^4 to 6.5×10^5)	1.2×10^6 (3.9×10^5 to 4.6×10^6)	9.6×10^6 (4.4×10^6 to 2.5×10^7)	2.6×10^7 (1.3×10^7 to 7.8×10^7)
SRB	8.9×10^4 (3.4×10^4 to 3.3×10^5)	1.2×10^5 (3.9×10^4 to 4.6×10^5)	NT[a]	NT

[a]not tested
[b]95% confidence interval

95% confidence intervals. Considering that the MPN method provides a one order of magnitude estimate of the population (de Man 1975) the MPN values for the H_2-utilizing methanogens were not very different. MBWIP-10A and MBWIP-10B had statistically similar MPN values for both the H_2- and acetate-utilizing methanogens (10^6/g).

The MPN method is generally held to be rather imprecise (Colwell 1979). However, of enumeration techniques, it is likely to give the best results because it involves the least perturbation of the organisms (Zinder 1993). Since the duplicate MPNs produced very similar values, confidence can be placed in the enumeration data reported for the fine tailings samples.

Methanogen MPNs are sometimes incubated at 37°C, shaking for 1 mon and then analyzed for methane in the headspace (Jain et al. 1991). Other protocols have different incubation procedures, and inoculated MPN tubes in this study were incubated stationary in the dark at room temperature for 3 mon. Methane analyses were performed on the August 1997 methanogen MPNs after 1, 2, 3, and 4 mon incubation. Since 3 mon incubation was used to enumerate the methanogens in the fine tailings samples, other values obtained after 1, 2 (Table 3.4 and 3.5) and 4 mon (data not shown) were compared to the 3 mon value. Seventeen of the twenty-six MPN values did not change between 1 and 3 mon incubation. However, in nine cases the MPN values increased after 1 mon and in these samples, less than 20% of the population (as indicated by the 3 mon MPN value) was detected after 1 mon incubation. Incubation for 2 mon was sufficient to enumerate the methanogenic populations with only one MPN value less than the value obtained after 3 mon incubation. Incubating the MPN tubes for longer than 3 mon had no effect on the MPN value. If time is a limiting factor, MPN tubes could be scored after 1 mon which would provide good approximation of the size of the methanogen population. Re-scoring at 2 or 3 months would be advised because methanogens are slow growers and values may change. To remain consistent with previous enumeration studies of fine tailings samples (Sobolewski 1992, 1999) the methanogen MPN tubes were incubated for 3 mon.

Table 3.4: Comparison of the MPN values determined for acetate-utilizing methanogens after 1 and 2 mon incubation at room temperature to the values collected after 3 mon for August 1997 samples. The critical MPN_1/MPN_2 ratio for statistical significance was 5.2.

Sample	1 month	2 months	3 months
MB1-1	2.70×10^{2a}	5.0×10^{3b}	9.90×10^{5}
MB1-5	ND[c]	ND	6.14×10^{6}
MB1-10	ND	ND	9.12×10^{6}
MB1-15	4.41×10^{5d}	9.20×10^{5b}	4.25×10^{6}
MB1-20	2.60×10^{5a}	1.60×10^{6b}	1.64×10^{6}
MB2-1	ND	ND	3.00×10^{3}
MB2-5	ND	ND	6.55×10^{5}
MB2-10	2.40×10^{4a}	2.10×10^{5b}	4.06×10^{5}
MB2-15	2.61×10^{4a}	3.1×10^{5b}	6.20×10^{5}
MB2-20	6.70×10^{4a}	6.50×10^{5b}	2.77×10^{6}
DP-5	ND	ND	5.44×10^{4}
MBIP1-1	ND	ND	9.00×10^{3}
MBIP1-5	ND	ND	1.59×10^{7}

[a]MPN value was statistically lower than 2 and 3 mon values
[b]value was not statistically lower than 3 mon value
[c]values are not statistically different from 3 mon values
[d]MPN value was not statistically different than the 2 mon value but was statistically lower than the 3 mon value

Table 3.5: Comparison of the MPN values determined for H_2-utilizing methanogens after 1 and 2 mon incubation to the MPN values collected after 3 mon for August 1997 samples. The critical MPN_1/MPN_2 ratio for statistical significance was 5.2.

Sample	1 month	2 months	3 months
MB1-1	1.40×10^{3a}	1.4×10^{3b}	9.00×10^{5}
MB1-5	ND[c]	ND	4.77×10^{6}
MB1-10	ND	ND	4.56×10^{6}
MB1-15	ND	ND	7.18×10^{5}
MB1-20	ND	ND	2.58×10^{6}
MB2-1	ND	ND	3.00×10^{3}
MB2-5	5.40×10^{5a}	1.20×10^{6b}	3.47×10^{6}
MB2-10	ND	ND	3.19×10^{5}
MB2-15	ND	ND	4.79×10^{5}
MB2-20	ND	ND	1.05×10^{5}
DP-5	6.50×10^{3a}	3.3×10^{4b}	1.05×10^{5}
MBIP1-1	ND	ND	2.30×10^{4}
MBIP1-5	ND	ND	2.86×10^{10}

[a]MPN value was not statistically different than the 2 mon value but was lower than the 3 mon value
[b]value was statistically lower than 3 mon value
[c]value was not statistically different from 3 mon value

3.2.2 Effects of prolonged sample storage on microbial populations

Sample collection occurred in the summer when access to, and sampling on, the MLSB was favorable. Samples required storage prior to microcosm experiments initiated throughout the year. Storage at 4°C was chosen, as microbial activity at this temperature would be depressed without being lethal to the bacterial populations, and because this temperature has been previously used by personnel at Syncrude Canada Ltd. There was some concern that storage might have an unanticipated effect on the microbial populations, which could alter methanogenesis and interfere in the interpretation of the results.

An experiment was designed to study the effect of the length and temperature of storage on samples collected in July 1998. Population size of acetate-utilizing and H_2-utilizing methanogens and SRB was monitored over time. Samples were stored at 4°C, 14°C and room temperature and were tested after 0, 1, 3, 6 and 9 mon storage.

MPN tubes were inoculated at each time point, enumerating acetate-utilizing (Figure 3.2) and H_2-utilizing methanogens (Figure 3.3) and SRB (Figure 3.4) for samples stored at 4 and 14°C. Generally there were no major changes in the three populations over time especially when considering the ranges of the 95% confidence limits (Figures 3.2 to 3.4). Values fluctuated and variability was noted but no consistent trend was evident. Despite the variability observed, the sizes of the populations did not drop with storage but remained in the MPN range of 10^4 to 10^6/g indicating that the numbers were not affected by storage.

MPN values of acetate-utilizing methanogens generally ranged (Figure 3.2) between 10^5 to 10^6/g with one count at 10^4/g (MBC2-15) and another at 10^7/g (MBWIP-10). MPN values of H_2-utilizing methanogens (Figure 3.3) had the same general 10^5 to 10^6/g range, with one value at 10^4/g (MBC2-15) and another at 10^7/g (MBWIP-10). SRB MPN values (Figure 3.4) typically ranged from 10^5 to 10^6/g with one value at 10^7/g (MBC1-5). All three populations were enumerated in the samples stored at room temperature only after 9 mon. The MPN values after room temperature storage did not deviate from the ranges noted for the samples stored at 4°C and 14°C.

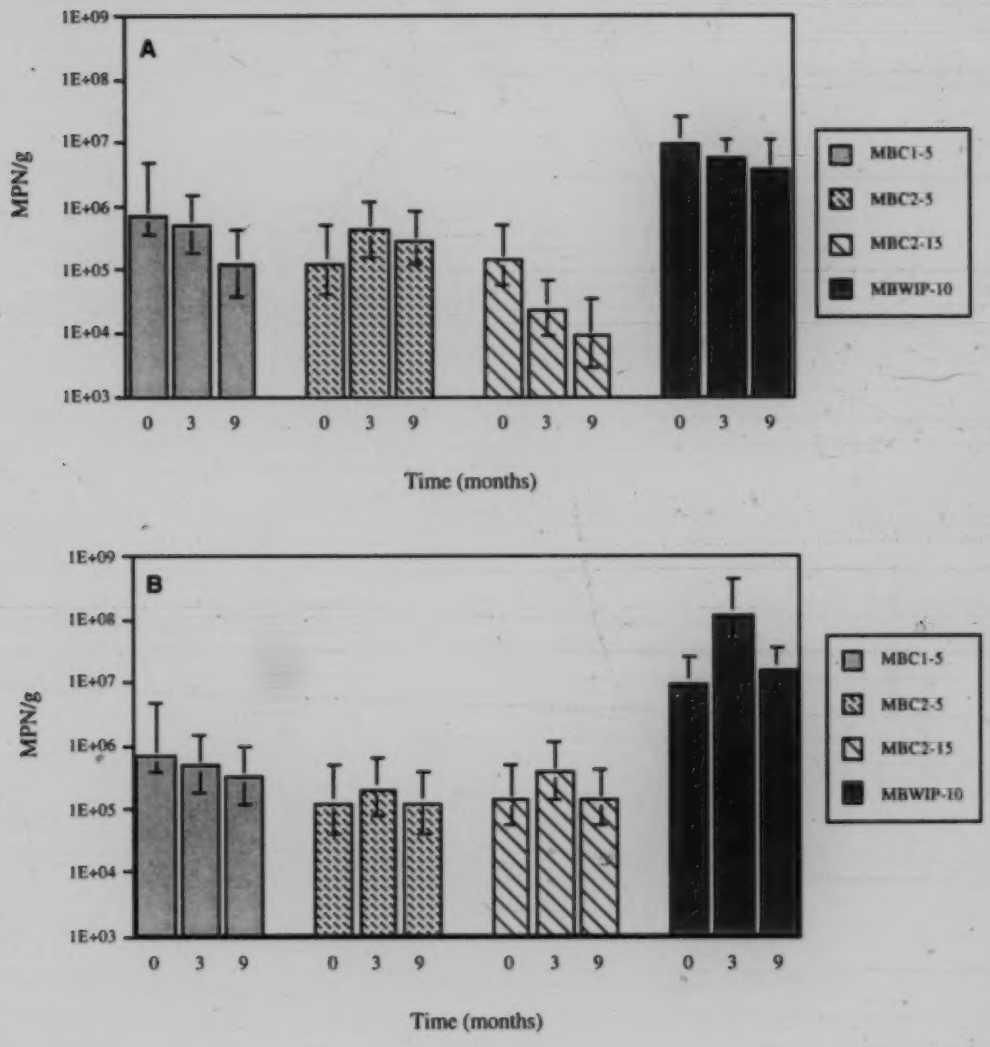


Figure 3.2: The MPN values for acetate-utilizing methanogens in samples stored at 4°C (A) and 14°C (B) for up to 9 mon. Bars represent 95% confidence limits.

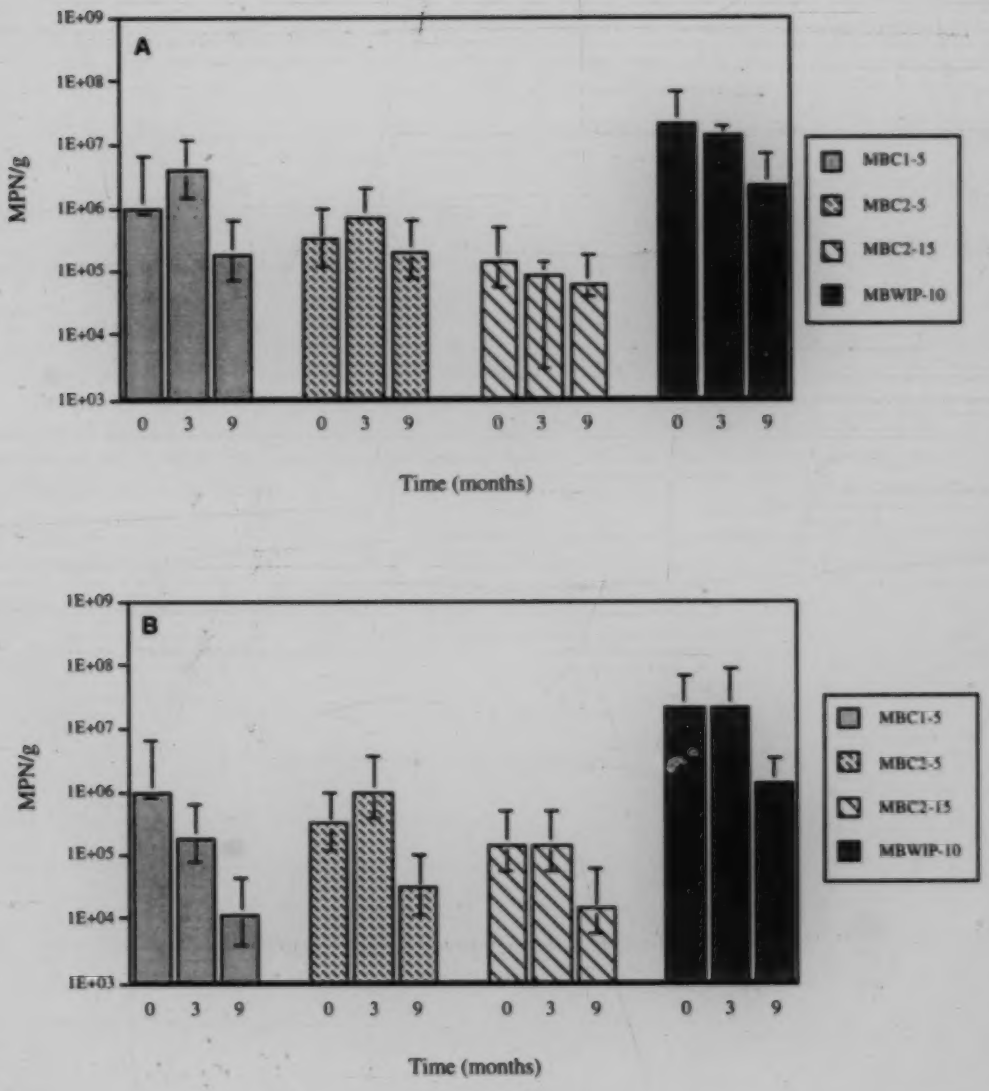


Figure 3.3: The MPN values for H_2-utilizing methanogens in samples stored at 4°C (A) and 14°C (B) for up to 9 mon. Bars represent 95% confidence limits.

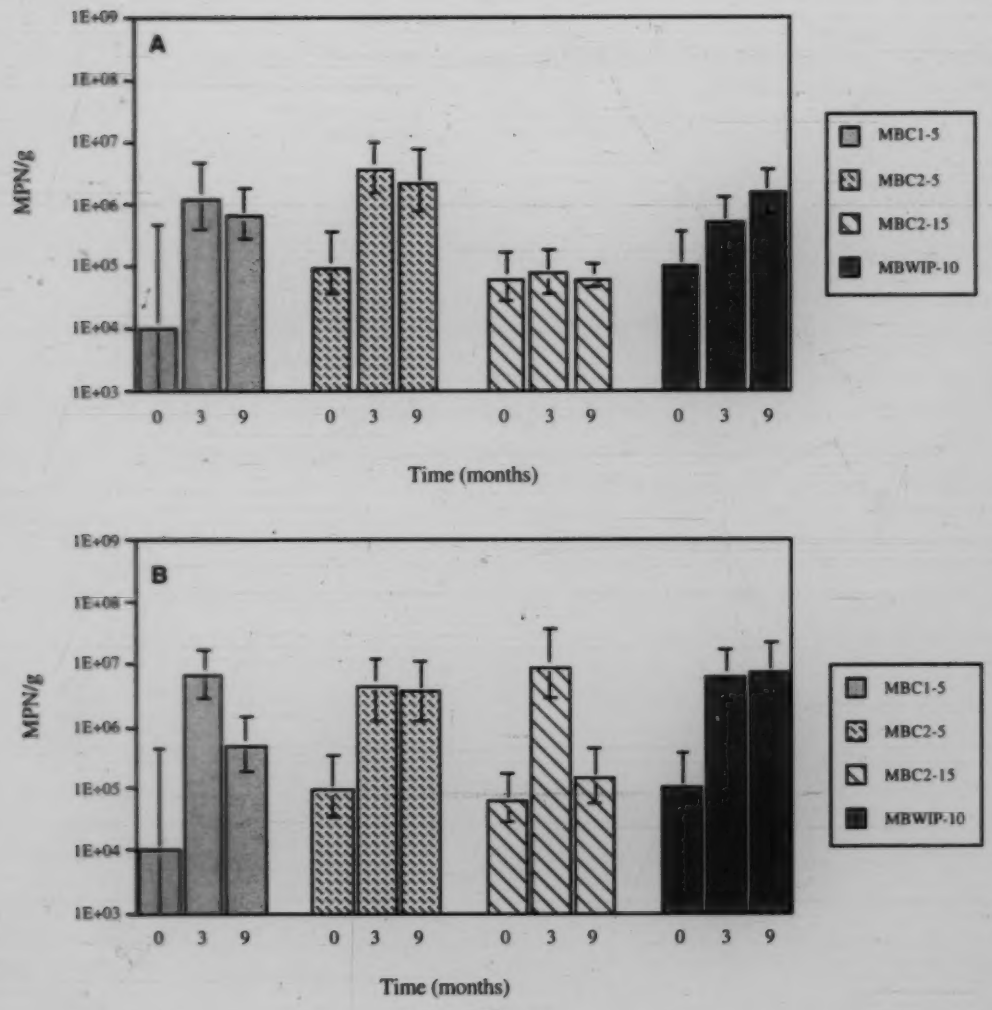


Figure 3.4: The MPN values for SRB in samples stored at 4°C (A) and 14°C (B) for up to 9 mon. Bars represent 95% confidence limits.

The MPN method of enumeration relies on the assumption that a homogeneous mixture is successfully serially diluted to extinction. It is this assumption that provides a reasonable explanation for the variability noted in the MPN values. The fine tailings samples are thick and viscous and it is possible that the samples were not mixed to the same degree in each situation and therefore the dilutions contained a heterogeneous mixture. As well, the addition of sample to the first dilution blank (2 mL sample to 18 mL medium) is likely to vary by $\pm$ 0.3 mL. This variability in initial dilution will affect the MPN values.

SRB values obtained from time 0 samples commonly had lower numbers than the MPN values at time 1, 3, 6, and 9 (Figure 3.4). It is possible that there was more stringent scoring biases employed at time 0 than at the other times. The end point for SRB, while simple, the reduction of resazurin from pink to clear and the formation of a black FeS precipitate, is somewhat arbitrary and susceptible to individual interpretation. Sometimes, the medium would not be reduced but a black precipitate would be formed, or the nails appeared to turn black but no other precipitate was found in the tubes or there would be settled solids massed at the bottom of the tube containing biofilm, inoculum and other debris. Scoring of these tubes was subjective and the standards of acceptance at time 0 may have been more stringent than at other times. Further support for this explanation is the fact that the methanogen scores did not have this variability from time 0 to other storage times. The end point scored for the methanogens is the production of methane; this is a much less arbitrary decision and is not as dependent on the subjectivity of the experimenter.

The data presented here indicate that prolonged storage of the fine tailings samples at 4°C, 14°C, or room temperature had little effect on the culturable population sizes of the SRB and the H_2- and acetate-utilizing methanogens. The relatively constant populations sizes over 9 mon indicate that there are enough nutrients and substrates available in the samples to maintain these microbial populations over a long-term and that new cells likely replenish the population minimizing the impact of cell death. The effect of storage on other microbial communities was not investigated. Storage may alter the population sizes of other

microorganisms in the consortium which could affect methanogenesis if substrate levels dwindle.

3.2.3 Methanogen enumeration in 1997 and 1998

Most methanogens are able to reduce CO_2 to CH_4 in the presence of H_2, but some use simple substrates such as formate, methanol, methylamine or acetate as methanogenic substrates (Jain et al. 1991). Consequently, it is important to test for the presence of different types of methanogens when doing enumeration studies as some species represent only a fraction of the total microbial community present in a sample. Therefore, all of the samples collected were enumerated for H_2- and acetate-utilizing methanogens. DP-1, 98DP-1 and 98DP-5 were omitted from statistical comparisons because the dilution for these samples was underestimated.

Of the four preliminary samples enumerated in July 1997, there were equal numbers of H_2- and acetate-utilizing methanogens. Similarly, in 18 samples collected in August 1997, 13 had numbers of acetate- and H_2-utilizing (Appendix H) methanogens that were the same, three had more acetate-utilizing methanogens and two had more H_2-utilizing methanogens. Of the samples collected in July 1998, seven had the same numbers of acetate- and H_2-utilizing methanogens (Appendix H), two samples had more acetate-utilizers and one sample had more H_2-utilizers. Due to the similarity between the two different methanogen types, only the acetate-utilizers have been reported and used for statistical comparisons.

Methanogens are synergistically associated with anaerobic bacteria in interspecies H_2 transfer, therefore one might have expected that there would have been more H_2-utilizing methanogens than acetate-utilizers (Schink 1997). An equal abundance of the two populations suggests that both acetate and H_2 are substrates in the fine tailings samples, and are likely being supplied to the methanogens as a by-product from the metabolism of larger compounds by other anaerobic populations (Figure 1.4).

3.2.3.1 Comparison of the numbers of methanogens

In 1997, the lowest methanogen MPN values were obtained from the samples collected from a depth of 1 m (Tables 3.6 and 3.7). Samples M1, MB1-1, MB2-1, and MBIP1-1 all had statistically similar MPN values (10^3/g). There were no significant differences among the MPN values of samples collected from 5 m to 20 m (10^5 to 10^6/g) within a given sample site or between sample sites MB1 and MB2. Nor were there differences in MPN values among the July samples taken from 5 to 15 m (10^6/g). There was little difference between the MPN values of methanogens detected in the July and August 1997 samples. Of the fine tailings samples, DP-5 had the lowest MPN value of methanogens (10^4/g) and MBIP1-5 had the greatest value (Table 3.7). Sample MBIP1-5 was enumerated twice. The first MPN value obtained (5.08×10^{10}/g) was considered uncharacteristically high. A repeat enumeration was performed on MBIP1-5 sample, which had been stored at 4°C for 7 mon. The second enumeration provided an MPN value of 1.59×10^7/g. This value is likely a better representation of the population size of the acetate-utilizing methanogens because storage of the sample did not likely affect the population size as noted previously (Figure 3.2). With an MPN value of 10^7/g, MBIP1-5 continues to have the largest methanogen population of all the samples collected in 1997.

Of the samples collected in 1998, the lowest methanogen MPN values were found in the 1 m water samples (10^3 to 10^4/g). The fine tailings sample collected from the Base Mine Lake (MBWIP-10) had the highest MPN value of methanogens (10^7/g) (Table 3.7). Of the 1998 samples collected, MBC1-5, MBC1-8 and MBC2-5 and MBC2-10 had statistically similar MPN values (10^6/g) which were higher than the other fine tailings samples enumerated (10^5/g).

Comparison of the 1997 and 1998 results show that the MPN values of methanogens in the water samples (1 m) did not change (10^3/g) and were the lowest of all the MLSB samples. The low numbers are consistent with the possible presence of dissolved oxygen in the water which is toxic to methanogens.

Table 3.6: Enumeration of acetate-utilizing methanogens in samples obtained from the MLSB in 1997 and 1998. Values are expressed as MPN/g dry weight except the 1 m surface water samples which are expressed as MPN/mL. The critical MPN_1/MPN_2 ratio for statistical significance was 5.2.

Depth (m)	July 1997 (M)	Aug 1997 site 1 (MB1)	Aug 1997 site 2 (MB2)	July 1998 site 1 (MBC1)	July 1998 site 2 (MBC2)
1	3.00×10^3	9.90×10^3	3.00×10^3	2.30×10^3	NS
5	1.88×106	6.14×10^6	6.55×10^5	1.02×10^6	1.17×10^5
8	NS[a]	NS	NS	1.24×10^{6}[b]	NS
10	4.25×10^6	9.12×10^6	4.06×10^5	7.77×10^4	9.20×10^5
15	4.79×10^6	4.25×10^6	6.20×10^5	NS	1.44×10^5
20	NS	1.64×10^6	2.77×10^6	NS	9.55×10^4

[a]site not sampled
[b]averaged value of two MPN assays

Table 3.7: Enumeration of acetate-utilizing methanogens in samples obtained from the Demonstration Pond and the Base Mine Lake in 1997 and 1998. Values are expressed as MPN/g dry weight except the 1 m surface water samples which are expressed as MPN/mL. The critical MPN_1/MPN_2 ratio for statistical significance was 5.2.

Depth (m)	Demonstration Pond 1997 (DP)	Demonstration Pond 1998 (98DP)	Base Mine Lake 1997 (MBIP1)	Base Mine Lake 1998 (MBWIP)
1	<20	<2	9.00×10^3	7.00×10^3
5	5.44×10^4	$>4.37 \times 10^5$	5.08×10^{10}[a]	NS
10	NS[b]	NS	NS	1.80×10^7

[a]repeat enumeration of a 7 mon old sample resulted in a MPN value of 1.59×10^7/g
[b]site was not sampled

While there was slight variability in the methanogen MPN values in the MLSB samples, overall, the 1998 samples did not differ significantly from the 1997 samples (10^5 to 10^6/g). There was little difference in the MPN values of methanogens among the various depths sampled on the MLSB (Table 3.6). Therefore, as the fine tailings settle and the various substances concentrate, there are no negative effects on the methanogens. The methanogenic population in the MLSB rose drastically from non-detectable levels in 1992 to MPN values of 10^5 to 10^6/g in 1996 (Sobolewski 1992, 1999). Methanogen MPN values in the MLSB have remained constant through 1997 and 1998 which may suggest that the population has reached a dynamic equilibrium around the MPN value range of 10^5 to 10^6/g. Continued enumeration studies will confirm this hypothesis.

While the methanogenic populations in the MLSB may have reached a steady state, MPN values have increased significantly in the fine tailings of the Demonstration Pond from 1997 (5.44×10^4/g) to 1998 ($>4.37 \times 10^5$/g) (Table 3.7). Subsequent enumerations will determine whether numbers will continue to rise. The Demonstration Pond is a pilot project for the wet landscape remediation plan and the low methanogen numbers and activity were promising. If methanogen numbers continue to increase, then the feasibility of water capping may be compromised, because methane in the water may facilitate the establishment of an active methanotrophic population. Methanotrophs will utilize the methane and deplete the water of oxygen and the resultant anoxic conditions may impact the viability of the lake ecosystem.

The Base Mine Lake samples MBIP1-5 (1997) and MBWIP-10 (1998) had similar methanogen MPN values (10^7/g). These values are significantly higher than those obtained by Sobolewski (1999), who collected samples at 5, 6 and 10 m in the Base Mine Lake in 1996 and reported MPN values from 10^3 to 10^5/g. The medium used by Sobolewski was slightly different in composition than the one used here however, both were bicarbonate-buffered and acetate as a carbon source. All other aspects of the two procedures were the same so comparing the two sets of data is acceptable. The 100-fold increase in methanogen

numbers in the Base Mine Lake between the enumeration by Sobolewski in 1996 and the 1997 samples is of considerable importance. The Base Mine Lake is the start of the full-scale wet landscape remediation plan. That methanogen numbers have risen quickly and are remaining at a level higher than the MLSB samples which may delay the creation of a self-sufficient lake ecosystem.

3.2.4 SRB enumeration in 1997 and 1998

SRB can use a variety of substrates including organic acids, alcohols, amino acids, aromatic and linear organic compounds (Zinder 1993). To isolate, culture and enumerate SRB from environmental samples, lactate is generally supplied as the carbon and energy source (Butlin et al. 1949; Hines et al. 1997). Lactate at a final concentration of 1000 mg/L (12 mM) was used for the SRB MPN determinations in this study.

In July 1997, the lowest SRB MPN value was detected in the 1 m sample (1.7 x 10^5/g) and the highest at 5 m (6.4 x 10^7/g), with the other fine tailings samples in the MPN value range of 10^5 to 10^6/g (Table 3.8). In August 1997, DP-1 had the lowest SRB MPN value (Table 3.9), and all the other samples had SRB MPN values close to 10^4/g, except for the samples obtained from a depth of 5 m, which had MPN values of 10^7/g (Table 3.8). Similar trends were noted in July 1998 with the highest SRB MPN values in MLSB samples at 5 and 8 m while all the other samples had statistically similar SRB MPN values (10^4/g).

SRB are strict anaerobes, and the lower counts obtained from the surface waters (1 m samples) are understandable, as there is likely some O_2 present in the overlying waters of the MLSB. The SRB were found in greatest abundance in the samples obtained from a depth of about 5 m in the MLSB (Table 3.8 and 3.9). This depth is just below the water:tailings interface which is the point of highest sulfate concentration (MacKinnon 1989). SRB need sulfate as a TEA for anaerobic respiration, therefore it is appropriate that the number of SRB are high at the depth where sulfate has been consumed.

Tailings samples from August 1997 had lower SRB MPN values (10^3 to 10^4/g) than those from July 1997 (10^6 to 10^7/g) (Table 3.8), with the exception of the samples at 5 m which had similar MPN values (10^7/g). However, SRB MPN values at 5 m dropped from 10^7/g to 10^5/g between August 1997 and July 1998, while the other fine tailings samples remained around 10^4/g. The drop in SRB MPN values cannot be attributed to lower sulfate concentration in the 1998 samples because these samples contained significantly more sulfate than the 1997 samples (Tables 3.1 and 3.2). The MPN values reported here fall in the range 10^4 to 10^7/g documented by Sobolewski (1999) suggesting that the SRB population is not declining but fluctuating. Despite the changing numbers, the trends have not changed; SRB are highest in samples where sulfate has been consumed.

3.2.4.1 Growth of SRB on various electron donors

Since the substrate range of SRB is diverse, electron donors other than lactate were used to enumerate the SRB. The MPN values of lactate-, H_2-, acetate- , propionate-, and benzoate-utilizing SRB in the fine tailings samples ranged between 10^4 to 10^5/g (Table 3.10). MBC2-5 had statistically more H_2- and propionate-utilizing SRB than acetate-utilizing SRB. MBC2-15 had no statistical differences among any of the values obtained.

The medium used in the MPN tubes contained yeast extract which likely provided a alternative carbon source. It is unlikely that the small amount of yeast extract can account for the large MPN values obtained, therefore the SRB were likely using the electron donors provided. A control containing only yeast extract should have been included. Confirmation of the ability of the SRB to utilize the substrates added could have been obtained by monitoring the loss of lactate, benzoate, acetate and propionate in the tubes.

These results imply that the SRB in the fine tailings are able to use lactate, H_2, benzoate, acetate and propionate, suggesting a diversity in the SRB population. They also indicate that there may be a variety of substrates available in the fine tailings for SRB. This is the first study using fine tailings sample from the MLSB to test substrates other than lactate for SRB utilization. H_2, benzoate, acetate and propionate have been used as electron

Table 3.8: Enumeration of SRB in samples from the MLSB in 1997 and 1998. Values are expressed as MPN/g dry weight except the 1 m surface water samples which are MPN/mL. The critical MPN_1/MPN_2 ratio for statistical significance was 5.2.

Depth (m)	July 1997 (M)	August 1997 site 1 (MB1)	August 1997 site 2 (MB2)	July 1998 site 1 (MBC1)	July 1998 site 2 (MBC2)
1	1.70×10^5	1.10×10^4	5.00×10^3	1.70×10^4	NS
5	6.40×10^7	1.57×10^7	8.86×10^6	1.02×10^5	8.99×10^4
8	NS[a]	NS	NS	4.54×10^5	NS
10	2.00×10^6	3.76×10^3	6.39×10^3	6.58×10^4	5.60×10^4
15	2.25×10^5	4.52×10^4	1.41×10^4	NS	6.34×10^4
20	NS	7.04×10^4	5.23×10^4	NS	4.20×10^3

[a]site was not sampled

Table 3.9: Enumeration of SRB in samples from the Demonstration Pond and the Base Mine Lake in 1997 and 1998. Values are expressed as MPN/g dry weight except the 1 m surface water samples which are MPN/mL. The critical ratio for statistical significance was 5.2.

Depth (m)	Demonstration Pond 1997 (DP)	Demonstration Pond 1998 (98DP)	Base Mine Lake 1997 (MBIP1)	Base Mine Lake 1998 (MBWIP)
1	<20	<2	2.80×10^4	7.03×10^7
5	7.42×10^3	1.37×10^4	8.90×10^6	NS
10	NS[a]	NS	NS	2.30×10^4

[a]site was not sampled

Table 3.10: Enumeration of SRB with different electron donors in two fine tailings samples obtained from MLSB in July 1998. Values are expressed as MPN/g dry weight. The critical MPN_1/MPN_2 ratio for statistical significance was 5.2.

	MBC2-5	**MBC2-15**
Electron Donor	MPN/g	MPN/g
Lactate	8.99×10^4	5.60×10^4
H_2	2.74×10^5	6.62×10^4
Benzoate	6.65×10^4	4.03×10^4
Acetate	5.08×10^4	3.74×10^4
Propionate	3.13×10^5	1.44×10^5

donors to study SRB from other environmental samples (Collins and Widdel 1986; Hines et al. 1997), as well as other electron donors including butyrate, caporate, ethanol, formate, succinate and pyruvate (Hines et al. 1997).

3.2.5 Comparison of the numbers of methanogens to SRB

Comparison of methanogen and SRB MPN values (Tables 3.6 to 3.9) indicated that 13 of the 18 samples collected in 1997 had more methanogens than SRB. The five remaining samples (S1, MB2-1, MBIP1-1, MB1-5, and MB2-5) had statistically similar MPN values of SRB and methanogens which coincides with the samples that had the greatest SRB populations. Comparison of the MPN values of methanogens and SRB indicate that 5 of the 10 samples collected in July 1998 had similar numbers. Only MBC1-1 had more SRB than methanogens and four samples (MBC1-5, MBC2-10, MBC2-20 and MBWIP-10) had more methanogens than SRB.

SRB out-numbered methanogens in the 1 m water samples or at shallow fine tailings depths which is consistent with the likely presence of O_2 (to which methanogens are more sensitive) and high sulfate levels (which provides a competitive advantage for the SRB at these depths). Methanogens out-numbered SRB in the deeper fine tailings samples where sulfate is not present .

In most samples, SRB and methanogens were had MPN values of 10^5/g. The co-existence of large SRB and methanogen populations even at depths at which the SRB have strong competitive advantages (sulfate is available) could suggest that there is an abundance of acetate and H_2 present to support both populations or that the two populations are using different substrates and are not in direct competition.

3.2.6 Enumeration of methanotrophs

Methanotrophs are aerobic, methane-oxidizing bacteria which limit the flux of methane in natural systems and particpate in ammonium oxidation and oxygen depletion. Hence, methanotrophs play a very important role in the ecology of freshwater, marine and

terrestrial ecosystems (Topp 1997). Methanotrophs are generally found when both methane and oxygen are present and numbers in soils, sediments, and water range between 10^3 to10^6 colony forming units/g (Topp 1997). Since the surface waters of the settling ponds are continually exposed to methane it is a prime habitat for methanotrophic bacteria. Enumeration of water samples for methanotrophs was intended to determine whether the activities of the methanogens in the fine tailings samples and the presence of methane in the water had facilitated the establishment of an active methanotrophic population and whether their numbers were increasing.

Visual assessment of all the MPN tubes on day 14 and day 30 showed no turbidity. Analysis of the headspace by GC showed no change in the concentration of CH_4 and no production of CO_2. The lack of growth in all five of the MPN series prepared, including the positive controls indicated that something was wrong with the assay. Methanotrophs are difficult to grow and problems arose in obtaining viable cultures from the freeze-dried stocks obtained from ATCC. Due to the difficulties noted and because of the many other experiments planned for this research project, no further attempts to enumerate methanotrophs were undertaken.

3.3 Effects of prolonged sample storage on acetate utilization

In addition to studying the effect of prolonged sample storage at 4°C, 14°C and room temperature on the SRB and methanogen populations (section 3.2.2), microcosm studies were initiated to study the effect of prolonged sample storage on acetate utilization under methanogenic conditions. Methane production was monitored in microcosms containing stored samples and water (controls) or 1000 mg/L acetate (treatments). Prior to storage (time 0), the addition of 1000 mg/L acetate resulted in the production of 8.3, 8.5, 7.8, and 7.6% vol CH_4, for samples MBC1-5, MBC2-5, MBC2-15 and MBWIP-5, respectively. These values correspond to between 80 and 89% of the expected 9.5% vol CH_4 to be produced, as per Buswell's equation.

Most of the acetate-supplemented microcosms produced over 80% of the expected amount of methane and this value did not diminish with storage time (Table 3.11). Not only was the ability of the samples to convert acetate to methane not impeded, the lag period prior to significant acetate-stimulated methanogenesis was not affected (Table 3.12). Measurements of headspace gas for methane occurred at 5 to 7 d intervals, so not all of the sampling days were consistent between each microcosm study. For comparison, the time difference between the last day acetate-supplemented microcosms had the same amount of methane as the unsupplemented controls and the first day when acetate-supplemented microcosms contained more methane than the unsupplemented controls were reported (Table 3.12). Methane production from added acetate was found to occur between 16 and 23 d after inoculation. Therefore prolonged storage had no adverse effect on acetate-stimulated methanogenesis in the fine tailings samples.

From the studies performed with samples stored at 4°C, 14°C and room temperature, it can be concluded that prolonged storage (up to 9 mon) did not drastically affect: the numbers of methanogens present (section 3.2.2), the ability of methanogens to use acetate (Table 3.11), or the lag time before available substrates were converted to methane (Table 3.12). Therefore, samples stored for a prolonged period of time can be used successfully in experiments without concern about biased results with respect to acetate-stimulated methanogenesis. Non-acetate-utilizing members of the microbial community, however, may be affected by prolonged storage. If some of these populations are involved in supplying acetate as a breakdown product from a more complex compound, then methanogenesis could be delayed, as methanogenic substrates would be unavailable.

3.4 Inhibition of methanogenesis

3.4.1 The effects of mixing active and inactive samples

This experiment was designed to explore whether there were factors present in an inactive sample that would inhibit methanogenesis of an active sample. Methane production

Table 3.11: Summary of the amount of methane produced above control values in the microcosms supplemented with 1000 mg/L acetate and inoculated with July 1998 samples stored for up to 9 mon at 4°C, 14°C and room temperature. The expected methane from 1000 mg/L acetate was 9.5 % vol.

Storage Temp (°C)	Sample	Storage Time			
		1 mon	3 mon	6 mon	9 mon
		% of expected CH_4	% of expected CH_4	% of expected CH_4	% of expected CH_4
22	MBC1-5	80	79	96	93
	MBC2-5	88	91	95	92
	MBC2-15	88	89	86	90
	MBWIP-10	84	74	91	87
14	MBC1-5	84	80	87	93
	MBC2-5	89	89	92	84
	MBC2-15	84	97	97	91
	MBWIP-10	73	85	79	93
4	MBC1-5	87	73	92	92
	MBC2-5	95	94	99	104
	MBC2-15	72	74	99	82
	MBWIP-10	83	81	99	97

Table 3.12: Summary of the lag time prior to acetate-stimulated methanogenesis in July 1998 samples stored for up to 9 mon at 4°C, 14°C and room temperature.

Sample	Storage Temp (°C)	Storage Time (mon)	Last sampling day when acetate-supplemented microcosms had the same amount of CH_4 as the unsupplemented controls	First sampling day when acetate-supplemented microcosms contained more CH_4 than the unsupplemented controls
all[a]	none[b]	0	11	19
all	4	1	10	17
all	14	1	10	17
all	22	1	10	17
all	4	3	11	18
all	14	3	11	18
all	22	3	11	18
all	4	6	12	19
all	14	6	12	19
all	22	6	12	19
MBWIP-10	4	9	9	16
others[c]	4	9	16	23
all	14	9	9	16
MBWIP-10	22	9	9	16
others	22	9	16	23

[a]refers to all samples tested: MBC1-5, MBC2-5, MBC2-15 and MBWIP-10
[b]samples were collected and used prior to long-term storage
[c]refers to samples MBC1-5, MBC2-5 and MBC2-15

production in microcosms containing a mixture of a non-active (DP-5) and active (MB1-15) samples was compared to methane production by microcosms containing either sample. Microcosms received no supplement (Figure 3.5A), acetate (Figure 3.5B) or H_2 (not shown).

When no supplement was present (Figure 3.5A), microcosms containing DP-5 produced significantly less methane than the microcosms containing MB1-15 or the mixture of the two samples. Consequently, mixing the two samples together did not alter methanogenesis of the active sample. Therefore there are no inhibitory components present in the DP-5 sample that can suppress methanogenesis.

When acetate was added, all three sets of microcosms produced the same amount of methane (Figure 3.5B). Identical results were seen with the H_2-supplemented microcosms (data not shown). These data suggest that the methanogens present in both the active and inactive samples are able to effectively utilize both acetate and H_2 and therefore the difference between the active and inactive designations is only based on the abundance of methanogens. Why does DP-5 (10^4/g) have fewer methanogens than MB1-15 (>10^6/g) if there are no strongly inhibiting compounds present *in situ*?

The chemistry of the Demonstration Pond water samples (Table 3.1 and Table 3.2) were markedly different from the MLSB waters. Perhaps the different ionic nature of the overlying water has influenced methanogenesis in the Demonstration Pond fine tailings samples. Sulfate concentrations are low at 5 m in the Demonstration Pond (6 to 9 mg/L, Table 3.1) thus the SRB are not likely limiting the substrate supply for the methanogens. The water cap may provide a source of Fe^{3+} for competing iron-reducing bacteria as Fe^{3+} usually enters anaerobic sediments by diffusion from the aerobic water layer (Zinder 1993). The DOC values in the 5 m fine tailings samples were 52 and 54 mg/L (Table 3.1) which is comparable to the DOC values in the other fine tailings samples, therefore, the amount of available substrates for methanogenesis is not likely depleted in the Demonstration Pond.

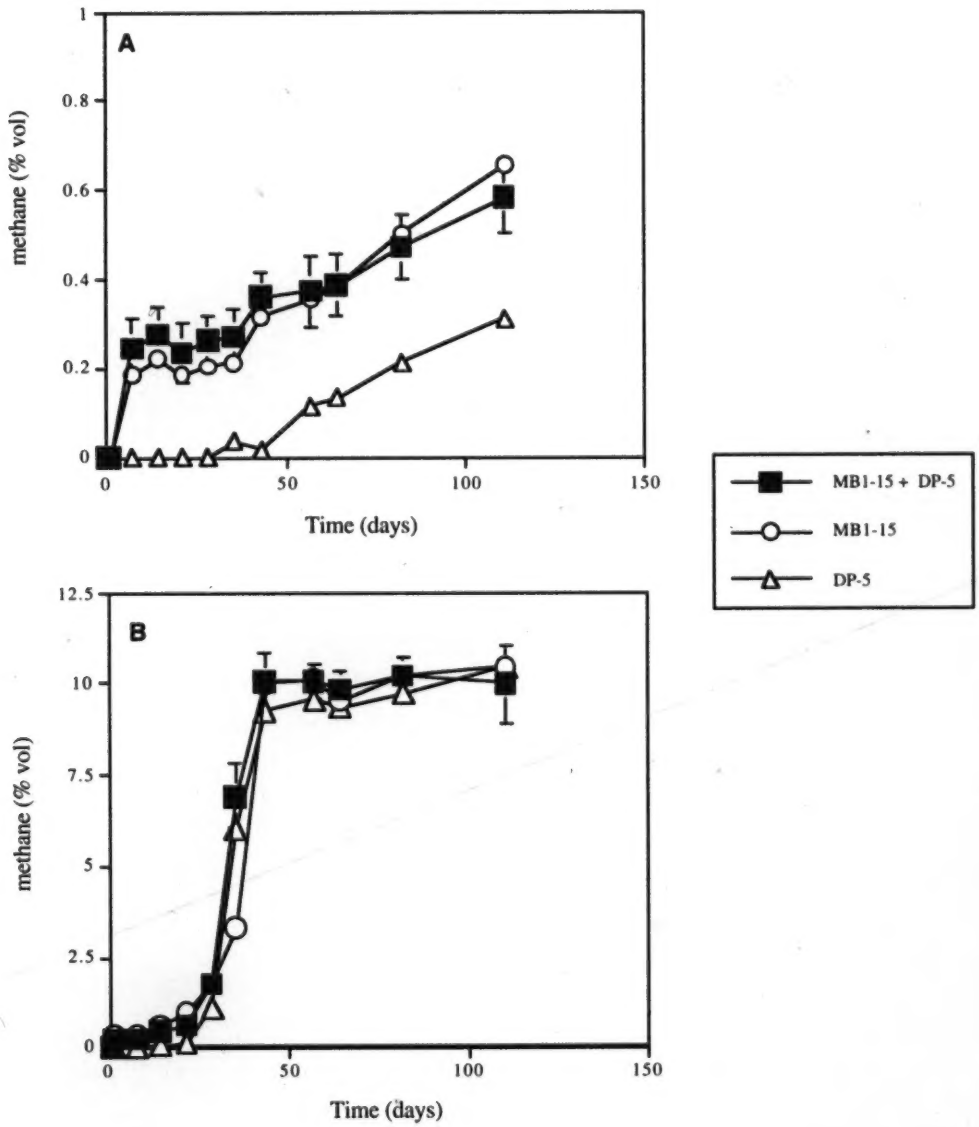


Figure 3.5: The effect of mixing an active (MB1-5) and an inactive (DP-5) fine tailings sample on methanogenesis. Microcosms were unsupplemented (A) or received 1000 mg/L acetate (B). Each point represents the mean of triplicate microcosms and bars for controls represent one standard deviation.

When the Demonstration Pond was established, methanogenesis in the fine tailings had not become a major concern. The fine tailing used to create the base of the Demonstration Pond probably had very low methanogen numbers. It is possible that the water cap has slowed the growth of the methanogenic population in the Demonstration Pond and therefore these samples do not have as active a methanogenic population as the MLSB samples.

3.4.2 Controlling methanogenesis by the addition of TEAs

The evolution of methane from the MLSB is undesirable. It was hypothesized that the addition of TEAs which yielded more energy for other microbial populations (Figure 1.4) would lead to competition for available electron donors causing a decrease in methanogenesis. To study this, nitrate and sulfate were the TEAs chosen.

3.4.2.1 The effect of nitrate addition

Londry and Suflita (1999) have reported that 50 mM (3000 mg/L) nitrate inhibited methanogenesis in sludge samples collected from a settling tank at a US Navy Fuel Depot (<0.1% methane produced in the inhibited microcosms). Preliminary work with the July 1997 samples (M5, M10, M15) demonstrated that 3000 mg/L nitrate inhibited methane production from day 1. However, because of the limited amount of sample, this study was done with only one microcosm per sample and so further work was required. In the subsequent experiment, the ability of 3000 mg/L nitrate to inhibit methanogenesis was tested, as were lower concentrations of 1800 and 600 mg/L.

Six different samples were used (Table 3.13) in this study and each sample produced the same trends of inhibiting methanogenesis as shown in Figure 3.6A, with the addition of 600, 1800 and 3000 mg/L nitrate. The time period before observation of statistically significant inhibition of methanogenesis (onset of inhibition; Table 3.13) was determined by using Dunnett's statistical method. The day of inhibition is denoted by the arrow in Figure 3.6B which shows an enlarged portion of the first 60 d of the experiment

for sample MB1-15. The time before inhibition varied between the different samples tested, ranging from 4 to 45 days (Table 3.13). However, the time of nitrate-induced inhibition for a given sample was found to be the same for all concentrations tested. For example, for sample MB1-15, addition of 3000, 1800 or 600 mg/L nitrate significantly inhibited methanogenesis by day 45 (Table 3.13, Figure 3.6B). The samples collected from 5 m had the shortest delay before inhibition of methanogenesis (4 to 30 d) whereas the samples from 15 m took longer (45 d) (Table 3.13).

Addition of 3000 and 1800 mg/L nitrate to fine tailings samples resulted in sustained inhibition with less than 1.0 % vol CH_4 produced in the microcosms. Addition of 600 mg/L nitrate inhibited methanogenesis, however, prolonged incubation allowed for a slight increase in methanogenesis (Figure 3.6A) which produced between 2.5 to 4.6% vol CH_4 in the microcosms (Table 3.13). The failure of methanogenesis to be restored in the microcosms containing 1800 mg/L nitrate suggests that there is a threshold concentration between 600 and 1800 mg/L nitrate at which methanogenesis is successfully inhibited without any resumption of methanogenic activity.

The inhibition of methanogenesis by the addition of nitrate occurs because nitrate is the TEA required by nitrate-reducers for anaerobic respiration. Nitrate-reducers are better able to compete for available substrates than methanogens, and therefore providing abundant nitrate gives the nitrate-reducers a large competitive edge (Holland et al. 1987; Large 1983). The recovery of methanogenesis in the microcosms containing 600 mg/L nitrate may indicate that all of the nitrate had been consumed and the methanogens have regained some competitive advantage. Similarly, sustained inhibition at the 1800 and 3000 mg/L concentration ranges suggests that the nitrate levels have not been depleted sufficiently to allow methanogens to successfully compete for available substrates.

Dissimilatory nitrate-reducers convert nitrate to nitrous oxide via nitrite in the process of denitrification (Atlas and Bartha 1993). Davidson (1991) stated that laboratory studies have shown that when the availability of nitrate is high relative to the availability of

Table 3.13: Summary of the time elapsed before statistically significant inhibition of methane production in microcosms with 600 mg/L nitrate inoculated with August 1997 fine tailings samples and incubated at 14°C.

Sample	Onset of statistically significant inhibition of methanogenesis (days)	Final % vol CH_4	Final % vol CH_4 (controls)
MB1-5	4	2.6	9.0
MB2-5	14	2.8	9.6
MB1-15	45	2.5	11
MB2-15	45	3.5	8.6
MBIP1-5	15	4.6	11
DP-5	30	2.7	6.5

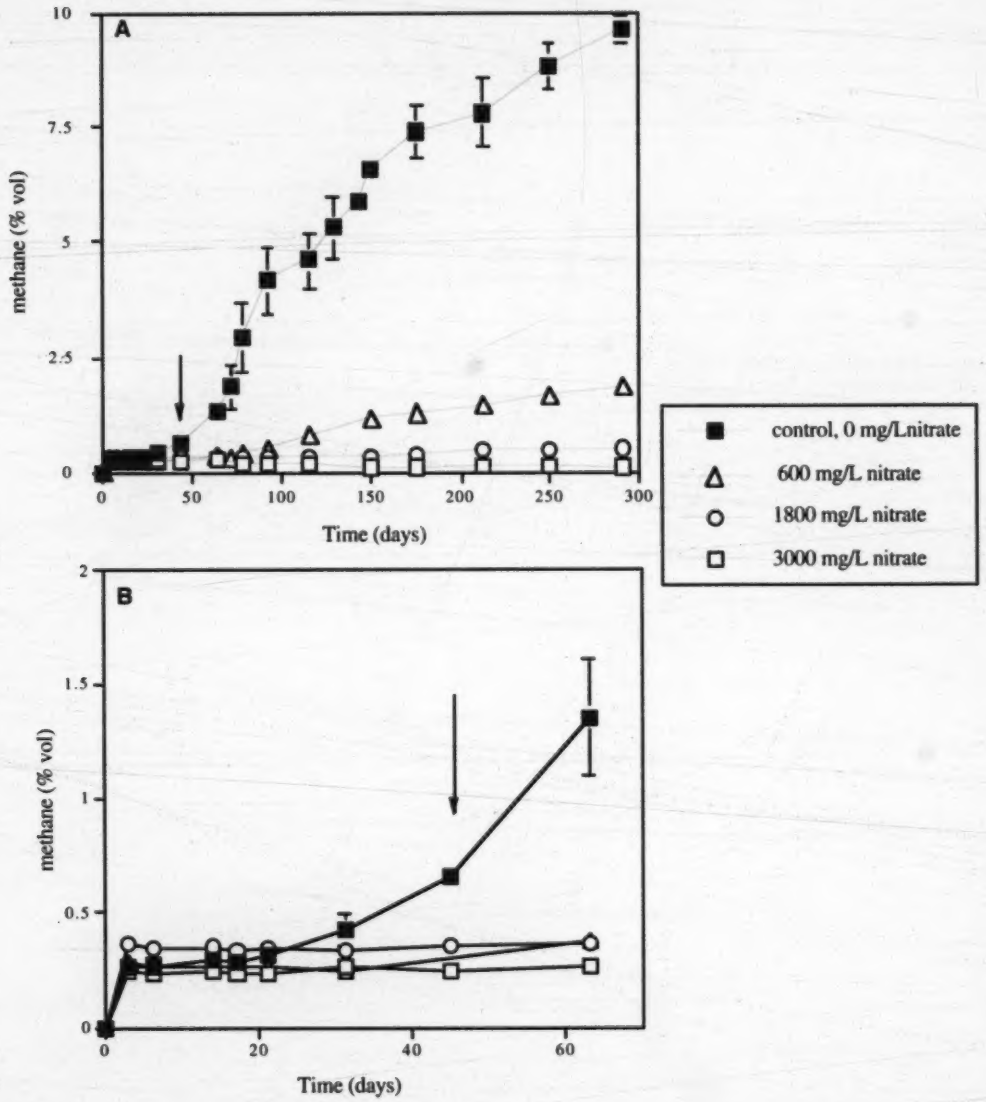


Figure 3.6: The effect of the addition of 600, 1800 and 3000 mg/L nitrate on methanogenesis after 290 d incubation (A), and an enlargement of the first 60 d of the experiment (B). Arrow indicates the date at which there was statistically significant inhibition of methanogenesis. Results from August 1997 fine tailings sample MB1-15 are representative of the other samples tested. Each point is the mean of triplicate microcosms and the bars for the control represent one standard deviation.

organic carbon, the primary endpoint of denitrification is nitrous oxide. Therefore to confirm that the inhibition was likely a result of bacterial competition, the microcosms were analyzed for the presence of nitrous oxide. Nitrous oxide was detected in all of the microcosms containing 3000 mg/L nitrate. Nitrous oxide was not found in the microcosms supplemented with 1800 and 600 mg/L but this was likely due to detection limits of the GC. Since nitrous oxide was detected in the microcosms containing the highest concentration of nitrate, indicating bacterial respiration, it is likely that denitrification also occurred in the microcosms containing 1800 and 600 mg/L nitrate.

Since sodium nitrate was used to provide the TEA to the microcosms there was some concern over the potential interference of the high concentrations of the sodium cation (220 to 1110 mg/L). Studies with anaerobic waste treatment processes have shown that sodium in concentrations up to 4600 mg/L (Kugelman and Chin 1971; Kugelman and McCarty 1964) had no effect on methanogenesis and concentrations reaching 3500 to 5000 mg/L sodium only had moderate inhibitory effects (McCarty 1964). The concentration of 1110 mg/L sodium added to the microcosms was well below the concentrations documented to inhibit methanogenesis. Consequently, the inhibition of methanogenesis noted in the nitrate-supplemented microcosms was due to the activities of the nitrate-reducing bacteria and not likely a result of cation interference.

3.4.2.2 The effect of sulfate addition

Londry and Suflita (1999) reported that 20 mM (2000 mg/L) sulfate inhibited methanogenesis in sludge samples collected from a settling tank at a US Navy Fuel Depot (<0.1% methane produced in the inhibited microcosms). The results from a preliminary study using July 1997 samples confirmed that sulfate at a concentration of 2000 mg/L inhibited methane production in the fine tailings. Inhibition occurred first in sample M5, next in M10 and finally in M15 (Table 3.14). The amount of methane in the controls, plateaued around 12%. The extent and onset of inhibition of methanogenesis by sulfate addition appeared to be related to the number of SRB present in the sample (Table 3.14).

The highest number of SRB was found in sample M5, in which inhibition by sulfate supplementation occurred most quickly. In contrast, sample M15 had the lowest number of SRB and the addition of sulfate had the least effect on methanogenesis.

The inhibition of methanogenesis by the addition of sulfate arises from the competition between SRB and methanogens for substrates such as H_2 and acetate. Because of the thermodynamics of these two processes, SRB are better competitors because they obtain more energy from the substrates than do the methanogens (Holland et al. 1987; Large 1983). One would predict that the larger the initial population of SRB, the sooner inhibition would occur, and this is what was observed. The delay before inhibited methane production in samples M10 and M15 was likely a result of the lower SRB numbers. In time, the population of SRB increased to a point where their rate of consumption of the substrates was high enough to reduce and eventually stop methanogenesis.

Due to a limited amount of sample, the preliminary study was not done in triplicate and so further confirmation regarding the inhibition of methanogenesis by sulfate was sought. In the subsequent experiment, the ability of 2000 mg/L sulfate to inhibit methanogenesis was confirmed and the effect of 5000 and 8000 mg/L sulfate were tested.

Each of the three concentrations of sulfate was tested on six different samples. The addition of sulfate (regardless of concentration) had sustained inhibitory effects on the samples collected at 5 m beginning from day 2 to 18 (Table 3.15). Methanogenesis was also inhibited by sulfate in samples collected at 15 m after 45 d. There was a slight increase in methanogenesis in MB1-15 microcosms containing 8000 mg/L sulfate (Figure 3.7A) which was also observed in MB2-15 (data not shown). Methanogenesis resumed at day 115 and continued for the next 40 d before methane levels plateaued.

The preliminary experiment with the July 1997 samples suggested that inhibition by sulfate was dependent on the number of SRB present in the sample and this was further confirmed (Table 3.15). Inhibition occurred quickly in samples with large SRB populations

Table 3.14: Summary of the effect of the addition of 2000 mg/L sulfate on July 1997 fine tailings samples versus the initial number of SRB present in the sample. Statistically significant inhibition of methanogenesis was determined by comparing methane values of unsupplemented controls to microcosms containing sulfate using Dunnett's method.

Sample	SRB/g	Onset of statistically significant inhibition of methanogenesis (days)	Final % vol CH_4	Final % vol CH_4 (controls)
M5	6.40×10^7	4	0.2	12.7
M10	2.00×10^6	7	1.5	12.1
M15	2.25×10^5	18	7.0	12.9

Table 3.15: Summary of the effect of the addition of 8000 mg/L sulfate on August 1997 samples. Statistically significant inhibition of methanogenesis was determined by comparing methane values of unsupplemented controls to microcosms containing sulfate using Dunnett's method.

Sample	SRB/g	Day of statistically significant inhibition of methanogenesis	Final % vol CH_4	Final % vol CH_4 (controls)	Sulfate consumed (mg/L)
MB1-5	1.57×10^{7a}	7	0.7	9.1	3620 ± 1400
MB2-5	8.86×10^{6a}	14	0.6	9.6	3000 ± 30
MBIP1-5	8.90×10^{6a}	18	1.1	10.8	3360 ± 340
MB1-15	2.25×10^{5b}	45	3.6	9.6	980 ± 780^c
MB2-15	4.52×10^{4b}	45	5.7	8.6	980 ± 730^d

[a]statistically similar values
[b]statistically similar values which are not similar to [a] values
[c]one replicate had only 250 mg/L loss of sulfate
[d]one replicate had only 200 mg/L loss of sulfate

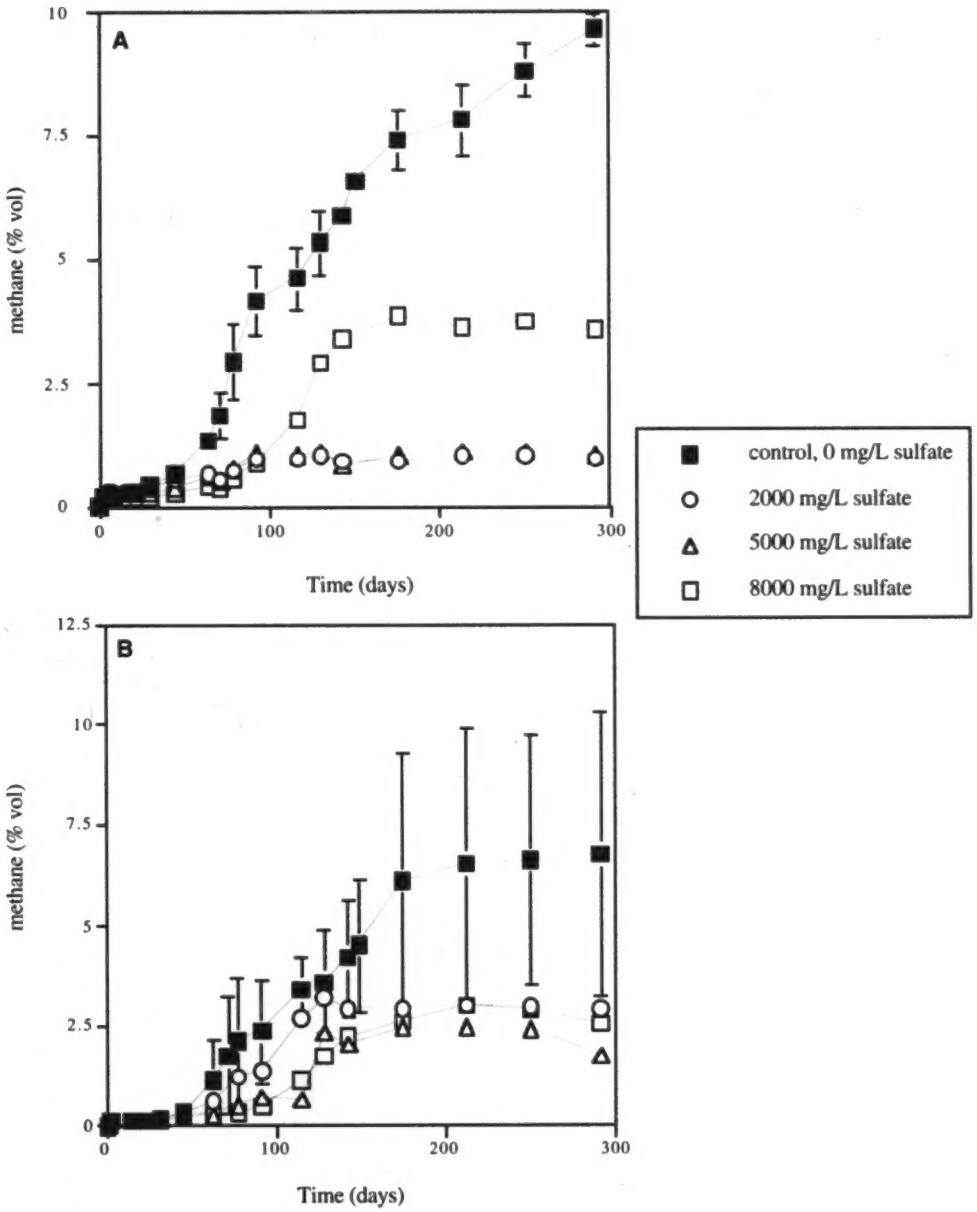


Figure 3.7: The effect of the addition of 2000, 5000 and 8000 mg/L sulfate on methanogenesis in August 1997 samples MB1-15 (A) and DP-5 (B). Each point is the mean of triplicate microcosms and bars for the control represent one standard deviation.

(MPN values $>10^6$/g) and took longer for samples with smaller SRB populations (MPN values $<10^5$/g).

Loss of sulfate in all the microcosms confirmed that bacterial competition was occurring which likely caused the observed inhibition in methanogenesis. Sodium was added as sodium sulfate in concentrations ranging from 725 to 2900 mg/L, which is below the 4800 mg/L tolerance limit documented for methanogens (Kugelman and Chin 1971; McCarty 1964). Therefore cation interference was not likely a factor contributing to inhibition of methanogenesis.

The results obtained from DP-5 are inconclusive. The control microcosms had large deviations, and one of the triplicates broke leaving only two replicates for statistical analysis (Figure 3.7B). Microcosms containing sulfate showed significant inhibition at day 30, however, after this date, the large standard deviations in the control values made it difficult to determine statistical significance of the treatment values. While the statistical significance of the data cannot be conclusively determined, the trends suggest that methanogenesis in sample DP-5 can be inhibited by the addition of sulfate, but that methanogenesis may be able to recover over time and the overall inhibition of methanogenesis noted does not compare to the immediate suppression seen in the other fine tailings samples.

As mentioned, the microcosms containing fine tailings samples collected from 15 m and supplemented with 8000 mg/L sulfate, had a slight increase in methanogenesis. This phenomenon was not noted with any of the other fine tailings samples nor did the increase occur in the microcosms supplemented with 2000 or 5000 mg/L sulfate. Sulfate was shown to be consumed in the 8000 mg/L sulfate-supplemented microcosms (Table 3.15), however, both MB1-15 and MB2-15 had one replicate which did not utilize much sulfate (200 to 250 mg/L). The lack of sulfate loss in these replicates could explain a corresponding increase in methane production however, MB1-15 had two replicates which demonstrated the increase in methane production and MB2-15 had an increase in all three

replicates. The microcosms were not shaken during incubation and this could produce microenvironments with depleted sulfate which might allow for methanogenesis to proceed. Despite the noted increase, methanogenesis was still significantly inhibited by the addition of 8000 mg/L sulfate in these fine tailings samples.

3.4.2.3 Comparison between the addition of nitrate and sulfate

As seen from the experiments presented, nitrate in concentrations ranging from 600 to 3000 mg/L (10 to 48 mM) and sulfate in concentrations from 2000 to 8000 mg/L (21 to 83 mM) can inhibit methanogenesis in the fine tailings samples. Presumably, the addition of these TEAs successfully stimulates the activities of anaerobic bacteria which out-compete the methanogen population for the available substrates. Nitrate ≥1800 mg/L (29 mM) and sulfate ≥ 5000 mg/L (52 mM) are the most effective concentrations resulting in inhibition that is sustained over time. Nitrate is a superior inhibitor to sulfate in the fine tailings samples, in that methanogenesis is inhibited immediately and requires a lower concentration.

Although nitrate and sulfate were both shown to efficiently inhibit methanogenesis in the fine tailings, use of these TEAs to prevent methanogenesis in the MLSB is not feasible. For inhibition to be successful, there would have to be sufficient mixing of the TEAs throughout the MLSB and considering the large volume of fine tailings contained in the MLSB this is not plausible. Furthermore, the amount of TEAs that would have to be added to the tailings pond would be enormous and would have significant financial costs associated with it. Addition of nitrate is not an attractive option because nitrate reduction produces nitrous oxide (N_2O). Nitrous oxide is a greenhouse gas and estimates suggest that it contributes 2 to 6% to the greenhouse gas effect as it is 100 times more effective than CO_2 in absorbing infrared radiation (Alexander 1999). Nitrous oxide is also very stable in the atmosphere and when it enters the stratosphere it is converted to nitric oxide (NO) which destroys ozone. The production of nitrous oxide is not a desirable outcome in the settling ponds.

Sulfate is being used in a new technology developed to minimize the large volume of tailings waste generated. Composite tailings (CT) is a process in which gypsum containing Ca_2SO_4 is added to fine tailings. Cation exchange between Ca^{2+} and Na^+ in the clays occurs which causes them to aggregate and increase the slurry viscosity (List and Lord 1997). Through the CT process, the fine tailings dewater relatively quickly which further consolidates the fine tailings. The water is recycled back to the extraction process as the CT deposit consolidates (List and Lord 1997). While the CT process appears to be a good method of reducing the volume of fine tailings, little is known with respect to the microbial ecology of these samples. It is not known whether the presence of sulfate will stimulate the SRB in the composite tailings and if this stimulation will cause methanogenesis to be inhibited. Furthermore, if methanogenesis is inhibited, will it be prolonged or will methanogenesis resume after the SRB have depleted the available sulfate, and if this is the case, how will the production of methane affect the CT? Studies currently in progress by Fedorak and co-workers will provide more information with respect to the microbial ecology of CT. For example, preliminary results have shown that most of the sulfides produced by the SRB form metal precipitates alleviating concerns regarding H_2S production and release.

3.4.3 Effect of molybdate on methanogenesis

Molybdate has been used to inhibit SRB (Banat et al. 1983; Winfrey and Ward 1983; Coates et al. 1996a, 1996b). By incubating microcosms with molybdate, it was hypothesized, that methanogenesis would increase because the SRB would be specifically inhibited. The effect of molybdate on methane production was monitored in six sets of microcosms (Table 3.16) under three different conditions: no supplementation, supplementation with methanogenic substrate (H_2 or acetate) in the presence or absence of molybdate. All of the samples produced the same trends and so sample MB1-5 is provided as a representative sample (Figure 3.8). Both H_2 and acetate supplementation stimulated methanogenesis in all of the samples (Table 3.16), generally after 30 d incubation (Figure

3.8A). Addition of molybdate condition resulted in the inhibition of methane production. In all samples, the microcosms containing acetate + MoO_4^{2-} and no supplements + MoO_4^{2-} had sustained inhibition after 4 to 20 d. The inhibition in microcosms containing H_2 + MoO_4^{2-} was not as severe, but the amount of methane produced was well below the control values (Table 3.16). The heat-killed sterile controls produced <0.02% vol methane.

The MB1-5 sample, incubated at room temperature (Figure 3.8B), produced the same trends as the microcosms incubated at 14°C (Figure 3.8A). Substrate supplementation enhanced methane production and MoO_4^{2-} addition suppressed methanogenesis. The only difference between the two sets of microcosms was that the rate of methane production in the microcosms incubated at room temperature was faster. For example, methane production in the control microcosms plateaued after 50 d when incubated at room temperature, but had not plateaued after 130 d when incubated at 14°C.

Molybdate has been shown to stimulate methanogenesis (Banat et al. 1983), consequently, the inhibition of methanogenesis with the addition of MoO_4^{2-} was contrary to expectation, therefore a second set of microcosms was established using sample MB1-5. Acetate and H_2 additions stimulated methanogenesis and the addition of MoO_4^{2-} inhibited methanogenesis (data not shown). Further confirmation was provided using sewage sludge inoculum. H_2 and acetate additions stimulated methanogenesis and MoO_4^{2-} inhibited it.

Molybdate is a specific inhibitor of SRB because it interferes with the ATP balance in the cell and, because it is stereochemically similar to sulfate, it may compete with transport (Puhakka et al. 1990). It was added to microcosms to suppress SRB activity and encourage the growth and activities of the methanogens. The results presented here demonstrated that MoO_4^{2-} can inhibit methanogenesis. Other studies have reported that 20 mM MoO_4^{2-}, the concentration used in these experiments, can inhibit methanogenesis in anaerobic reactor sludge (Puhakka et al. 1990), and digestor waste (Karhadkar et al. 1987). Although MoO_4^{2-} can be used as a specific inhibitor for SRB, when it is introduced into a consortium it is difficult to predict what will happen.

Table 3.16: Summary of the total % vol CH_4 produced by August 1997 samples supplemented with acetate or H_2 and with or without molybdate. Microcosms were incubated at 14°C for 140 d.

Sample	Final CH_4 (% vol) in treatment microcosms					
	no substrate (control)	Acetate	H_2	no substrate + MoO_4^{2-}	Acetate + MoO_4^{2-}	H_2 + MoO_4^{2-}
MB1-5	9.7	14.9	18.7	0.50	0.42	8.6
MB2-5	7.6	13.3	17.2	0.31	0.28	3.6
MB1-15	10.4	16.0	19.5	0.33	0.36	10.8
MB2-15	9.9	16.8	15.4	0.43	0.37	3.1
DP-5	7.7	14.8	16.0	0.12	0.11	0.20

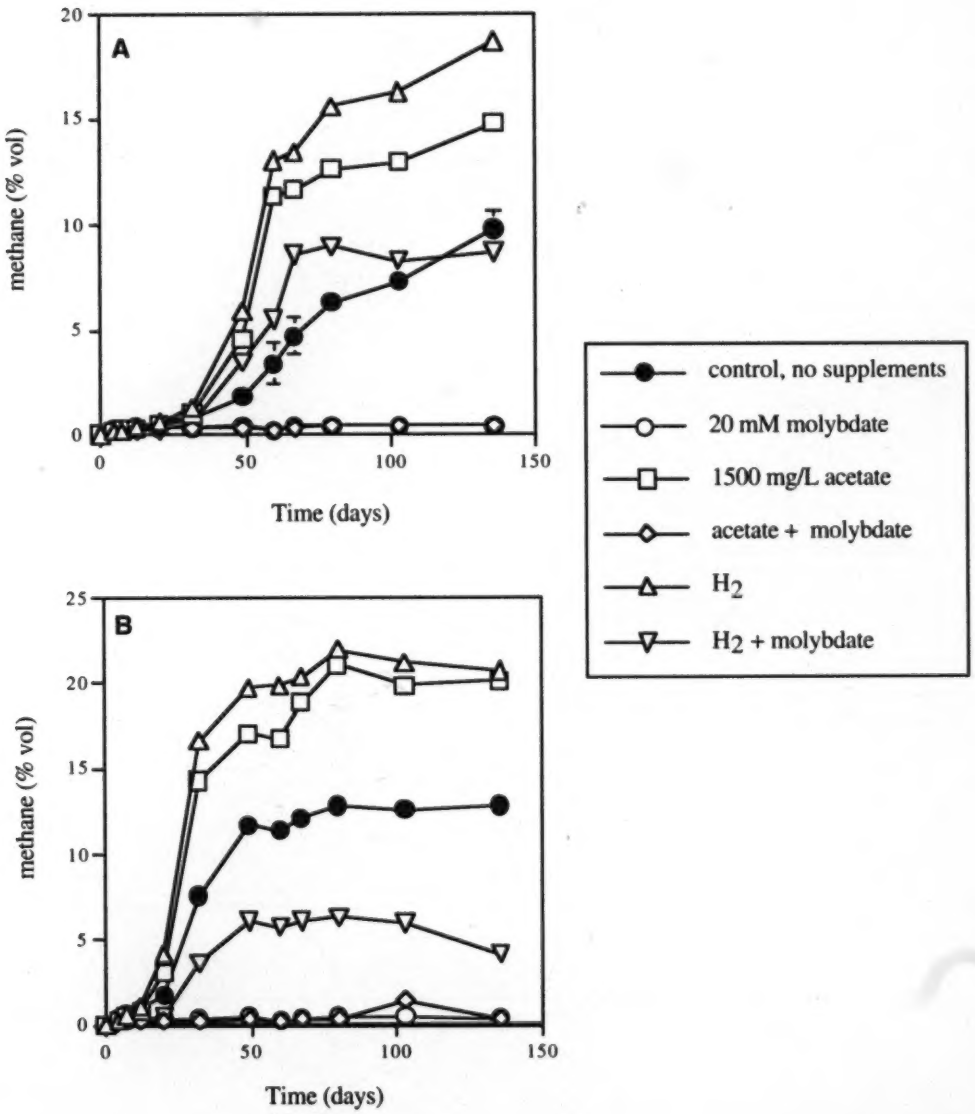


Figure 3.8: The effect of molybdate on methanogenesis in microcosms containing August 1997 sample MB1-5 and supplemented with acetate or H_2. Sample MB1-5 is representative of the other fine tailings samples and was incubated at 14°C (A) and at room temperature (B). Each point is the mean of triplicate microcosms and bars for the controls represent one standard deviation and are smaller than the symbol.

It has been suggested that the formation of MoO_2S^{2-} and $MoO_2SO_4^{2-}$ complexes limit the amount of free sulfide needed by methanogens for metabolic activities thereby limiting growth (Karhadkar et al. 1987; Puhakka et al. 1990). Furthermore, MoO_2S^{2-} and $MoO_2SO_4^{2-}$ complexes can interfere with enzyme activity either by blocking enzyme synthesis or by producing inactive proteins (Wolin and Miller 1980). In some of the samples, the H_2-supplemented microcosms were not as affected by the addition of MoO_4^{2-} ,= as the acetate-supplemented microcosms, agreeing with reports of Puhakka et al. (1990) that MoO_4^{2-} retarded the rate and extent of methane production and inhibited the growth of acetate-utilizing methanogens more so than H_2-utilizing methanogens.

Molybate was initially added to the microcosms containing fine tailings in order to specifically inhibit the SRB present with the intention of stimulating methanogenesis. It was anticipated that it could be shown that limiting the activities of the SRB in the fine tailings samples would facilitate methanogenesis as the methanogens would have a competitive advantage and obtain available substrate. Since the molybdate failed to be a specific SRB inhibitor and adversely affected the methanogenic populations as well, such conclusions cannot be made.

3.5 Potential substrates for methanogenesis

Seeking to determine what substrates were supporting methanogenesis in the fine tailings, microcosm studies were initiated. Potential substrates were added to microcosms containing fine tailings and it was expected that if the added compound was utilized by the consortium to produce methanogenic substrates then methanogenesis would be stimulated over controls which lacked the substrate supplement. Potential substrates tested included: petroleum, aromatic compounds and NAs. Prudhoe Bay crude oil and oil sands bitumen were the sources of petroleum tested. Aromatic compounds included a mixture of PACs, phenols, and ^{14}C-labelled toluene, naphthalene and phenanthrene. A variety of experiments using NAs were established to test the potential of these compounds to be methanogenic

substrates. NAs extracted from the surface water of the MLSB, a commercial mixture and surrogate NAs were different sources of NAs used in microcosm studies.

3.5.1 Petroleum

3.5.1.1 Potential for methane biogenesis from Prudhoe Bay crude oil

Microcosms, containing 50 mL inoculum, were supplemented with 20 μL of Prudhoe Bay crude oil. After 300 d incubation at room temperature, there were no significant differences in methane production between control and supplemented microcosms (data not shown). Prudhoe Bay crude oil did not stimulate or inhibit methanogenesis, so while there do not appear to be any substrates available for methanogenesis in the crude oil, there were not any inhibitory compounds either. It is possible that both inhibition and stimulation of methanogenesis was occurring concurrently resulting in no observable effect. The fine tailings in the MLSB contain on average 2% (w/v) bitumen (FTFC 1995a), therefore the consortium is likely adapted to the components of crude oil. Hence no observable effect on methanogenesis was noted after the addition of the crude oil supplement.

3.5.1.2 Potential for methane biogenesis from bitumen

The fine tailings stored in the MLSB contain on average 2% (w/v) (≈20 g bitumen/L fine tailings) residual bitumen (FTFC 1995a). Thus the potential of compounds contained in bitumen to stimulate methane production was investigated. It was hypothesized that if some component in the bitumen was a substrate for methanogenesis, the addition of more bitumen to the microcosms would yield more methane. To test this, 250, 750 or 2000 mg bitumen were added microcosms containing July 1998 samples MBWIP-10 or MBC1-8.

The addition of more bitumen did not affect methane production in sample MBWIP-10 (Figure 3.9A), but significantly inhibited the production of methane by MBC1-8 (Figure 3.9B). The relative standard deviations amongst the replicate microcosms were particularly high for both sample sets. The deviations noted in MBWIP-10 microcosms were 28, 11,

14, and 12% for the controls, 250, 750 and 2000 mg bitumen-supplemented treatments, respectively, and the relative standard deviations noted in the MBC1-8 microcosms were 4, 84, 30 and 15% for the controls 250, 750 and 2000 mg bitumen-supplemented treatments, respectively, as measured after 187 d incubation.

Methanogenesis was inhibited in sample MBC1-8, whereas added bitumen had no effect on methane production in MBWIP-10. MBWIP-10 is a more active sample than MBC1-8, as indicated by the amount of methane produced by the control microcosms. Perhaps the addition of bitumen did not affect methanogenesis in the microcosms containing MBWIP-10 because it was a more robust sample. The large deviations between the replicates made it difficult to detect small differences amoung the various treatments. It is possible that the deviations amongst the replicates was due to the amount of bitumen that was stuck to the neck of the serum bottle during inoculation or due to an incomplete mixing of the bitumen throughout the microcosms.

The microcosms contained 25 mL of fine tailings sample. Since there is ≈20 g bitumen/1000 mL fine tailings, then each unsupplemented microcosm contained ≈500 mg bitumen. The addition of 2000 mg/L bitumen gave a 4-fold increase in bitumen concentration in the microcosms. Thus, if some component of bitumen was a methanogenic substrate, addition of 2000 mg/L bitumen should have increased methane production 4-fold. This increase should have been detectable, yet none was observed.

The addition of bitumen to the microcosms may not have stimulated methanogenesis if the fine tailings samples were already saturated with bitumen. If bitumen was providing methanogenic substrates, then stimulated methanogenesis may not have occurred until *in situ* concentrations of bitumen dropped. The high variability amoung the replicates made detecting differences between the treatments difficult but stimulation should have been detected if substrates were available in the bitumen. Under the experimental conditions tested, oil sand bitumen did not stimulate methanogenesis.

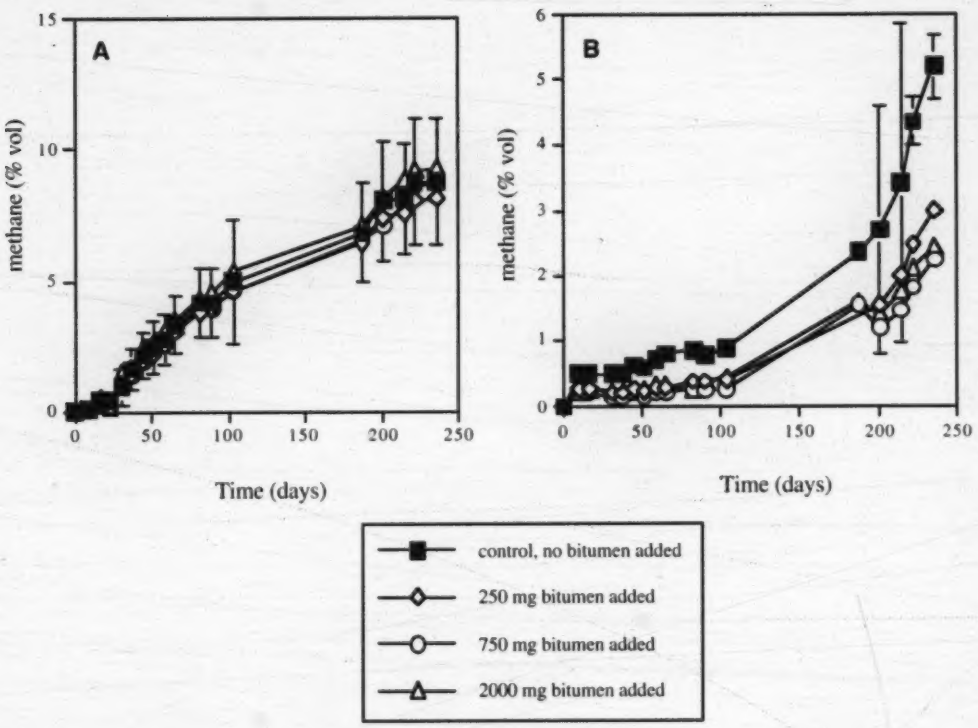


Figure 3.9: The effect of added bitumen on methanogenesis in July 1998 samples MBWIP-10 (A) and MB1-8 (B). Microcosms were incubated at room temperature. Each point represents the mean of triplicate microcosms and the bars for the controls represent one standard deviation.

3.5.2 Aromatic Compounds

3.5.2.1 Potential for methane biogenesis from a mixture of PACs

The exposure of fine tailings to a mixture of PACs including 1.5 mg each of: naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, dibenzothiophene, phenanthrene, fluorene, fluoranthene, biphenyl and 4-methylbiphenyl and 1 mg benzothiophene plus <1.5 mg anthracene, did not have any effect on methanogenesis after 300 d incubation (data not shown). There were no significant differences between the amount of methane produced by the control microcosms and those that received the mixture of PACs. Methanogenesis was neither hindered nor enhanced by the presence of the PACs, therefore, the consortium can withstand exposure to these compounds but does not readily utilize them in the production of methane.

3.5.2.2 Potential for anaerobic mineralization of ^{14}C-aromatic hydrocarbons

Using radiolabelled naphthalene, methyl naphthalene, phenanthrene, fluorene and fluoranthene, Coates et al. (1997) were able to detect $^{14}CO_2$ production from hydrocarbon-contaminated sediment samples after 40 d incubation under sulfate-reducing conditions. Since the fine tailings samples are continually exposed to hydrocarbons (Madill et al. 1999) the potential exists for the anaerobic microbial consortium to mineralize various aromatic hydrocarbons.

Despite the observation that methanogenesis had not been stimulated by a mixture of PACs (section 3.5.2.1), a study using individual radiolabelled compounds (^{14}C-labelled toluene, naphthalene and phenanthrene) was initiated. The mixture of PACs used in the first study contained a variety of different compounds, and it is possible that one or more components were toxic to the consortium while other components were stimulatory and these competing effects could result in no observable change in methane production. Testing single compounds would indicate whether individual aromatics were susceptible to degradation under methanogenic conditions. Furthermore, using ^{14}C-labelled compounds increases the sensitivity of detection as high specific activities can be added which better

imitates *in situ* concentrations and reduces any potential toxic effects caused by high concentrations. If $^{14}CO_2$ and $^{14}CH_4$ are detected then there is concrete evidence that the labelled compound has been degraded to provide methanogenic substrates.

Microcosms received 75 mL inoculum, 10 mL medium and one of the radiolabelled compounds (Table 2.2) and were sealed with a composite stopper. One set of microcosms was incubated under methanogenic conditions, and a second set received sulfate.

Toluene, naphthalene and phenanthrene were chosen because they are readily available commercially and the compounds increase from simple one-ring structures to two- and three-ring structures. Furthermore, toluene has been shown to be degraded under sulfate-reducing conditions (Coates et al. 1996), as have naphthalene and phenanthrene (Coates et al. 1997). Under methanogenic conditions, ^{14}C-toluene has been degraded to $^{14}CH_4$ (Coates and Lovley 1998; Grbic-Galic and Vogel 1986) but there are no reports of the mineralization of naphthalene and phenanthrene to methane.

In addition to the ^{14}C-labelled aromatics, ^{14}C-labelled hexadecane, a straight chain alkane was also tested. Until recently, hexadecane was thought to be recalcitrant under anaerobic conditions. However, ^{14}C-hexadecane has been shown to be mineralized under both methanogenic (Coates and Lovely 1998) and sulfate-reducing conditions (Coates et al. 1997; Coates and Lovely 1998).

Two sets of positive controls were prepared using ^{14}C-acetate and ^{14}C-hexadecanoic acid, both of which are degraded under methanogenic conditions (Jeris and McCarty 1965; Mah and Smith 1981). Hexadecanoic acid, also known as palmitic acid, is an intermediate in the aerobic degradation of hexadecane. Activity was detected in both sets of positive controls containing ^{14}C-acetate and ^{14}C-hexadecanoic acid (Table 3.17). The radioactivity, detected as $^{14}CO_2$ and $^{14}CH_4$ from the positive controls, did not increase with further incubation.

The values for % label recovered as $^{14}CO_2$ and $^{14}CH_4$ were determined by analyzing 1 mL headspace gas samples via the previously described ^{14}C-trapping system (section

2.9.9). The dpm/mL headspace value obtained was multiplied by the headspace volume of the microcosms to provide a value for total radioactivity (dpm) in the headspace. This total radioactivity value was divided by the radioactivity originally added to the microcosms to obtain a value for % label as $^{14}CO_2$ or $^{14}CH_4$. Carbon dioxide partitions between the gas and liquid phases and because the microcosms were not acidified to drive the CO_2 into the gas phase, a significant portion of the $^{14}CO_2$ was likely in the form of $H^{14}CO_3^-$ and not detected. The pH of the microcosms is near 7, and at this pH only 20% of the CO_2 is partitioned in the gas phase (Fedorak et al. 1982). As a result the detected $^{14}CO_2$ values are lower than the amount of label actually mineralized to $^{14}CO_2$. Methane does not partition in the liquid phase, therefore the $^{14}CH_4$ levels detected are accurate. In the positive controls 2 to 12 % of the label was mineralized to $^{14}CH_4$. Detection of 2% of the label as $^{14}CH_4$ produced 600 dpm/mL headspace. If the mineralization of the other ^{14}C-labelled compounds were to release less than 2% of the label as $^{14}CH_4$, the radioactivity would still be detected, as the system was able to detect radioactivity as low as 10 dpm/mL headspace.

Two sets of microcosms were initially established, one set was considered methanogenic and the second set was considered sulfate-reducing, because sulfate was added to each of the latter microcosms. At the first analysis time (44 d) it was noted that the sulfate-reducing positive controls had produced similar amounts of $^{14}CH_4$ as the methanogenic cultures (Table 3.17). To confirm that the label being trapped was $^{14}CH_4$, a headspace sample was injected into a GC and the CH_4 peak was separated from the CO_2 peak, and was flushed directly into ACS fluor. The activity counts obtained from the trapped CH_4 peak were between 30 and 68% of the activity counts obtained from the analysis via the trapping system. Previous work (Appendix G.2) had shown that radioactivity is lost as the sample travels through the GC, so the recovery values are reasonable. The radioactivity detected was $^{14}CH_4$. Furthermore, the headspace in the microcosms had over 20% vol CH_4. As there were no notable effects of sulfate addition after d 44, the two sets of microcosms were not considered significantly different.

Sulfate concentrations dropped by day 44 with further loss occurring after day 96 (Table 3.18). Large standard deviations were observed in the sulfate values. In these cases, one replicate had a high sulfate concentration while the second replicate had a low sulfate concentration. The variability between the replicates suggest that within the same sample, there are different rates of sulfate reduction. The SRB consumed a majority of the sulfate before the first analysis (44 d) of $^{14}CH_4$ and $^{14}CO_2$ and these microcosms had became methanogenic. Total % vol CH_4 (336 d) showed that methane levels were not lower in the sulfate-amended microcosms. Had these microcosms been re-amended with sulfate, methanogenesis would have been inhibited until sulfate levels dropped. It is unlikely that added sulfate would have resulted in the mineralization of the ^{14}C-compounds, because degradation did not occur initially when sulfate levels were high, and the depletion of sulfate was not used to degrade the ^{14}C-aromatic hydrocarbons.

The microcosms containing ^{14}C-toluene, naphthalene, phenanthrene and hexadecane were incubated for 336 d and there was no recovery of $^{14}CO_2$ or $^{14}CH_4$ under either the methanogenic or "sulfate-reducing" conditions. Despite the exposure of the fine tailings samples to aromatic compounds *in situ* (Madill et al. 1999), the consortium was unable to mineralize relatively simple aromatic hydrocarbons including toluene, phenanthrene and anthracene under the experimental conditions used. Furthermore, hexadecane did not stimulate methanogenesis within the fine tailings samples and therefore long chain alkanes are not likely the main substrate for methanogenesis in the fine tailings samples. The microcosms were monitored for 336 d and so any readily available substrates in the inoculum would have had time to be depleted. Consequently, if the ^{14}C-labelled compounds could support methanogenesis, $^{14}CH_4$ and $^{14}CO_2$ would have been detected.

Table 3.17: The percent of ^{14}C-label detected as $^{14}CO_2$ and $^{14}CH_4$ in headspace samples from microcosms containing ^{14}C-acetate and ^{14}C-hexadecanoic acid under methanogenic and "sulfate-reducing" conditions as determined after 44 d incubation at room temperature. ± represents one standard deviation from the mean value.

		Methanogenic conditions		"Sulfate-reducing conditions"	
Sample	^{14}C-labelled Compound	% label as $^{14}CO_2$	% label as $^{14}CH_4$	% label as $^{14}CO_2$	% label as $^{14}CH_4$
MBC2-15	acetate	0.21 ± 0.24	11 ± 14	0.75 ± 0.21	7.5 ± 2.6
	hexadecanoic acid	0.72 ± 0.13	4.1 ± 1.0	0.57 ± 0.38	2.3 ± 1.5
MBWIP-10	acetate	0.45 ± 0.07	12 ± 0.35	0.37 ± 0.05	3.0 ± 1.4
	hexadecanoic acid	0.68 ± 0.07	3.9 ± 0.30	0.75 ± 0.07	2.0 ± 0.50

Table 3.18: Sulfate concentrations over time in microcosms prepared to stimulate sulfate-reducing conditions in microcosms prepared to monitor for the mineralization of ^{14}C-aromatic hydrocarbons. ± represents one standard deviation from the mean value.

Sample	^{14}C-labelled Compound	Day of analysis	sulfate in treatments (mg/L)[a]	sulfate in sterile controls (mg/L)[b]
MB2-15	acetate	0	2560[c]	2760
		44	230 ± 190	1880
		96	480 ± 490	2360
	hexadecanoic acid	0	2054 ± 420	2340
		44	443 ± 46	1570
		96	91[c]	2020
MBWIP-10	acetate	0	2015 ± 120	2400
		44	0[d]	1270
		96	363 ± 230	2080
	hexadecanoic acid	0	1950 ± 155	2730
		44	0[d]	1400
		96	90[c]	1690

[a]mean of duplicate microcosms
[b]one heat-killed sterile control microcosm was prepared for each condition
[c]only one sample was available for analysis
[d]sample was diluted too much before analysis

3.5.2.3 Potential for methane biogenesis from phenols

Low concentrations of phenolic compounds including phenol, cresols, catechol and hydroquinone (Hargesheimer et al. 1984; Sobolewski 1992) have been detected in the MLSB. The ability of phenols to support methanogenesis has been widely studied and many reports exist on the successful utilization of phenol with corresponding production of methane (Tarvin and Buswell 1934; Fedorak and Hrudey 1984; Godsy et al. 1983; Wang et al. 1989). Cresols tend to be more resistant to degradation under methanogenic conditions, but degradation has been reported. Some researchers report complete mineralization of all three cresol isomers (Kaminski et al. 1990), whereas others report preferential metabolism of the *m*- and *p*-cresols (Smolenski and Suflita 1987; Wang et al. 1989). *o*-Cresol is the isomer most resistant to microbial attack under methanogenic conditions. However, the degradation of phenolic compounds is highly-dependent on the inoculum used. Microcosms were supplemented with phenol, *m*-cresol, *p*-cresol, or *o*-cresol to determine if the consortium could produce methane from these compounds.

3.5.2.3.1 Studies with August 1997 samples

Each of the samples tested were found to convert phenol to methane (72 to 86% recovery of expected methane) with the exception of DP-5 (Table 3.19). *m*-Cresol and *p*-cresol were also shown to stimulate methanogenesis in the fine tailings samples with the production of 52 to 86% and 53 to 93% of the expected methane, respectively. Increased methane production by phenol, *m*-cresol and *p*-cresol was directly correlated with loss of the phenolic compounds within the microcosms and re-supplementation of the microcosms resulted in further increases in methane production (Figure 3.10). One exception noted was MB1-15 which had moderate loss of the *m*- and *p*-cresols without methane production.

Conversion of the cresols to methane was as efficient as phenol but phenol stimulated methanogenesis more quickly between 69 and 76 d (Table 3.19). The order of *m*- and *p*-cresol conversion to methane differed between samples with methanogenesis occurring after 110 d. The shorter lag before mineralization of phenol is likely due to its

simpler chemical structure. Phenol is known to be a readily utilizable substrate for methanogenesis. Studies with anaerobic sewage sludge (Fedorak and Hrudey 1984) and continuous flow fermenters demonstrated mineralization of phenol within 15 d of inoculation (Wang et al. 1989). The long lag times (70 to 150 d) before stimulated methane production from phenol in the microcosms was surprising. At the time of inoculation the fine tailings samples had been stored for 8 mon which, at the time, was thought to affect both the activity and size of some of the anaerobic populations which may have contributed to the long lag periods. (This experiment was completed before the experiments testing the effect of storage on fine tailings samples (section 3.2.2) were initiated.)

If phenol, *m*-cresol, or *p*-cresol are present in the tailings samples, the consortium within the fine tailings will be able to use any of them to support methanogenesis. The inability of sample MB1-15 to mineralize *m*-cresol and *p*-cresol may suggest that heterogeneity of the MLSB is such that there are areas (or pockets) which are lacking the appropriate organisms or conditions needed to degrade *m*- and *p*-cresol or that the breakdown products are not suitable substrates for methanogenesis.

In contrast, *o*-cresol did not stimulate methanogenesis despite the depletion of 25 to 75% of the compound in the microcosms. In some cases, addition of *o*-cresol was noted to slightly inhibit methanogenesis but not with statistical significance. Since *o*-cresol was depleted in the microcosms, it is likely that other bacterial communities are transforming the *o*-cresol to compounds that cannot support methanogenesis such as 3-methylbenzoic acid (Bisaillon et al. 1991). The metabolism of *o*-cresol by other populations may engage the organisms which would normally provide substrates to the methanogens. This could potentially explain the minor inhibition of methanogenesis noted in the *o*-cresol-supplemented microcosms.

Table 3.19: Conversion of phenol and cresols to methane in microcosms containing August 1997 fine tailings samples and incubated at room temperature.

Sample	Substrate	Substrate used (%)	CH_4 Prod'n	% of Expected CH_4[a]	Days before enhanced CH_4 prod'n
MB1-5	phenol	100	+[b]	72	76
	m-cresol	100	+	52	142
	p-cresol	100	+	68	142
	o-cresol	18	no[c]	13	NS[d]
MB1-15	phenol	100	+	86	107
	m-cresol	45	no	30	NS
	p-cresol	55	no	58	NS
	o-cresol	37	no	2	NS
MB2-5	phenol	100	+	81	69
	m-cresol	100	+	86	107
	p-cresol	100	+	93	142
	o-cresol	45	no	38	NS
MB2-15	phenol	100	+	73	76
	m-cresol	100	+	65	128
	p-cresol	100	+	65	114
	o-cresol	57	no	0	NS
DP-5	phenol	100	no	69[c]	NS
	m-cresol	100	+	75	150
	p-cresol	100	+	97	142
	o-cresol	53	no	0	NS
MBIP1-5	phenol	100	+	83	69
	m-cresol	100	+	67	87
	p-cresol	100	+	53	142
	o-cresol	40	no	0	NS

[a]expected volume of methane to be produced was calculated using Buswell's equation, percentages values are based on CH_4 (% vol) in microcosms not mL CH_4 produced and are therefore approximations
[b]statistically significant stimulation of methane production
[c]no statistically significant effect on methane production
[d]methane production was not stimulated during experiment

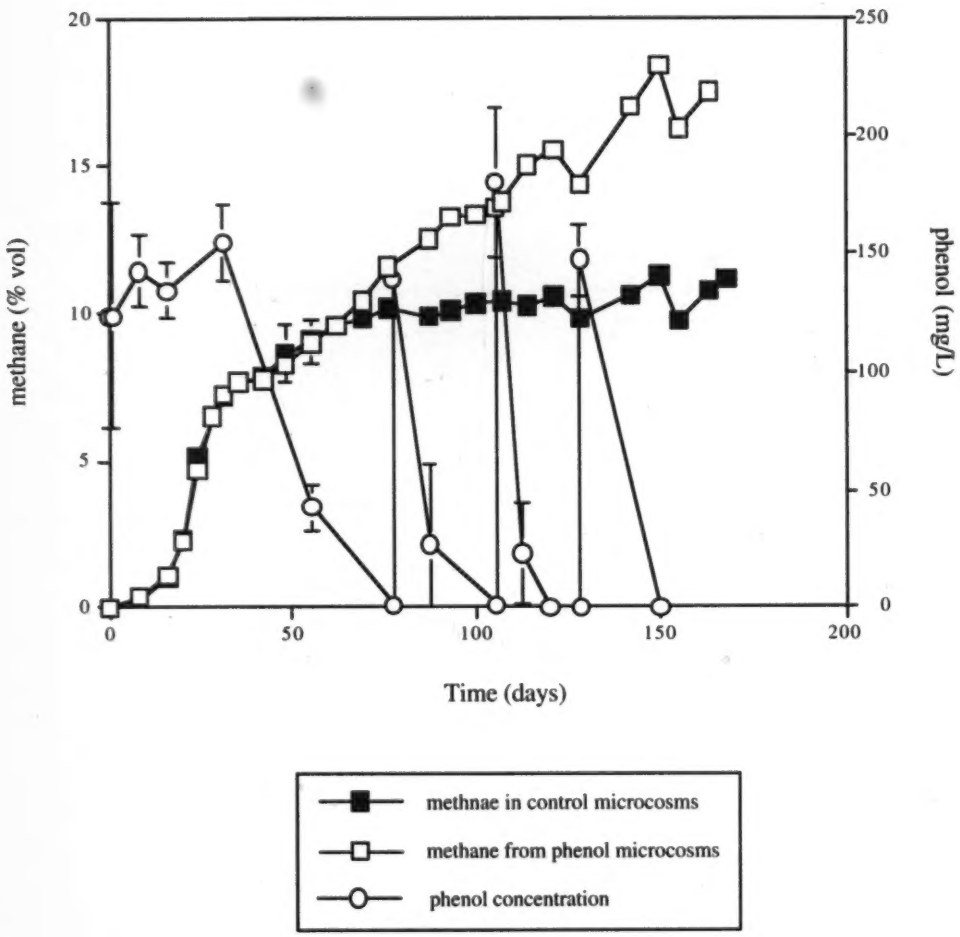


Figure 3.10: Methane production and depletion of phenol in microcosms containing August 1997 sample MB1-5 and incubated at room temperature. The control received no phenol. Microcosms were re-supplemented on days 79, 102 and 132. Each point represents the mean of triplicate microcosms. Bars for the phenol microcosms and controls (smaller than the symbol) represent one standard deviation.

Two sets of microcosms containing DP-5 or MBIP1-5 were incubated at 14°C, as this is close to the mean year-round temperature of the fine tailings in the MLSB (M. MacKinnon, personal communication; Table 3.1). There was no loss of any of the phenols and there was no stimulation of methanogenesis within 180 d incubation. There is no clear reason why mineralization of the phenols stalled when incubated at 14°C.

After inoculation and throughout incubation, a majority of the MBIP1-5 microcosms incubated at 14°C had the solids settle out to create two distinct layers, a medium layer and a fine tailings layer. This was unusual because generally, the fine tailings sample coated the internal surface of the serum bottle and remained as a suspension. The standard deviations associated with methane values from these replicates were also high which was attributed to these unusual microcosms. Consequently, the lack of degradation of phenols by this sample may be attributed to the character of the microcosms themselves. Temperature considerations are important and cannot be ignored but it is not clear, at this time, what effect temperature had on the degradation of phenols by fine tailings samples.

3.5.2.3.2 Studies with July 1998 samples

A second experiment designed to monitor the loss of phenols in microcosms with the subsequent production of methane was established using freshly collected fine tailings samples (10 days old) and was monitored for 180 d. The purpose of this study was to determine whether the long lag times noted in the first experiment involving phenols was due to the age of the inoculum.

Consistent with the first experiment, phenol, *m*-cresol and *p*-cresol were converted to methane by the fine tailings samples whereas *o*-cresol did not stimulate methanogenesis (Table 3.20). There was minimal substrate loss in the two sets of microcosms (MBC2-5 and MBC2-15) incubated at 14°C but there was no stimulation of methane production (data not shown). Acetate stimulated methanogenesis after lag times of 15 to 28 d in the microcosms incubated at room temperature (Table 3.20, Figure 3.11) and after 59 d in microcosms incubated at 14°C (Figure 3.11).

The phenols-supplemented microcosms were incubated >90 d before methanogenesis was stimulated, first by phenol and then by *m*-cresol and *p*-cresol generally after 120 to 150 d incubation. Overall, lag times before stimulated methane production occurred ranged between 98 and 155 d (Table 3.20). It was thought that the lag times before stimulated methane production would be shorter in the microcosms inoculated with recently collected fine tailings samples. Contrary to expectation, the lag times for the second experiment (98 to 155 d) were generally longer than the first (70 to 150 d). When compared, 11 of the 15 cases in which stimulation was successful in both experiments, lag times were shorter in the microcosms with the aged samples. It is possible that at the time of inoculation, the aged samples had less indigenous substrates than the freshly collected samples. Substrates would have been metabolized during the 8 mon storage at 4°C. Less substrate present in the inoculum may have allowed the consortium in the aged samples to degrade the phenols sooner than in the fresh samples.

The levels of the expected volume of methane produced was generally high (>75%) and there was a slight correlation between the length of lag time and the amount of methane produced (Table 3.20). That is. as lag times increased the percentage of expected methane produced dropped. For example, *m*-cresol stimulated methanogenesis after 122 d in sample MBC1-8 and produced 77% of the predicted methane whereas *m*-cresol stimulated methanogenesis after 155 d in sample MBC1-5 and only produced 40% of the expected methane (Table 3.20). The variability in the amount of methane produced by the phenols indicates that the ability of the fine tailings sample to mineralize phenol, *m*-cresol and *p*-cresol to methane, is inoculum and substrate dependent.

Loss of the phenolic substrates was correlated with increased methane production and after substrate concentrations were depleted, re-supplementation resulted in repeated methane stimulation (Figure 3.12). One exception was the phenol-supplemented microcosms which initially demonstrated loss of substrate without stimulated methanogenesis (Figure 3.13). These microcosms received their first re-supplementation

Table 3.20: Conversion of phenol and cresols to methane microcosms containing July 1998 fine tailings samples incubated at room temperature. Dry volume of methane is adjusted to STP.

Sample	Substrate	Substrate used (%)	CH_4 Prod'n	Produced CH_4 (mL)	% of Expected CH_4	Days before enhanced CH_4 prod'n
MBC1-5	phenol	100	+[a]	8.4	84	98
	m-cresol	100	+	2.1	40	155
	p-cresol	100	no[b]	0.8	15	NS[c]
	o-cresol	56	no	0.6	23	NS
	acetate	100	+	4.8	96	28
MBC1-8	phenol	100	+	9.8	98	85
	m-cresol	100	+	6.1	77	122
	p-cresol	100	+	2.4	91	136
	o-cresol	73	no	0	0	NS
	acetate	100	+	4.7	94	22
MBC2-5	phenol	100	+	2.5	50	172
	m-cresol	100	+	4.5	85	122
	p-cresol	100	+	4.5	85	115
	o-cresol	78	no	-0.4	0	NS
	acetate	100	+	2.1	84	15
MBC2-15	phenol	100	+	6.7	67	98
	m-cresol	100	+	4.8	91	136
	p-cresol	100	+	1.8	68	129
	o-cresol	62	no	-0.9	0	NS
	acetate	100	+	1.8	72	15
MBWIP-10	phenol	100	+	2.2	44	129
	m-cresol	100	+	3.9	74	122
	p-cresol	100	+	4.0	76	129
	o-cresol	65	no	-0.3	0	NS
	acetate	100	+	2.0	80	22

[a]statistically significant stimulation in methane production
[b]no statistically significant effect on methane production
[c]methane production was not stimulated

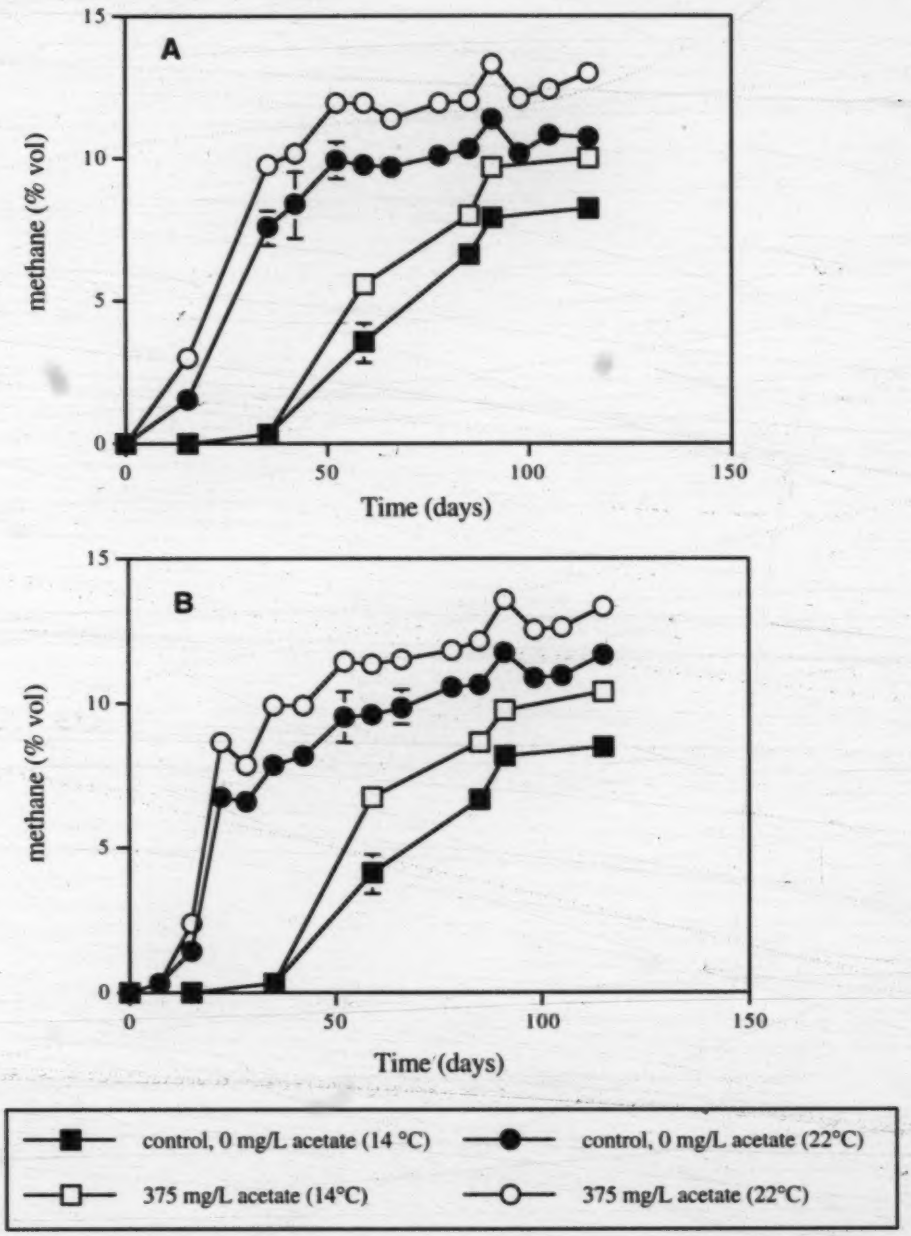


Figure 3.11: Methane production in microcosms containing 375 mg/L acetate and inoculated with July 1998 samples MBC2-5 (A) and MBC2-15 (B) and incubated at 14°C and at room temperature (22°C). Each point represents the mean of triplicate microcosms and bars on the controls represent one standard deviation.

before methanogenesis had been stimulated, however subsequent substrate loss resulted in concurrent methane production. Since initially there was loss of phenol without methane production it was suspected that anaerobic bacteria were out-competing the methanogens for the acetate from the breakdown of the phenol compound. Competition under anaerobic conditions is highly dependent on the concentration of appropriate TEAs and since methane production was stimulated after re-supplementation and with further incubation (Figure 3.13) it is likely that there was a decrease in the TEA required for the competing bacteria.

SRB were considered the likely competitors, as the sulfate concentrations in the inoculum were quite high (17 to 87 mg/L) with the exception of MBWIP-10 (2 mg/L) (Table 3.2). Analysis of sulfate in microcosms containing MBC1-5 and MBC2-5 indicated that by the end of the experiment none of the microcosms contained more than 3 mg/L sulfate, a significant decrease from the initial concentration of 43 mg/L. (The inoculum contained 87 mg/L sulfate and the microcosms would have 43 mg/L as a result of the 1:1 dilution with medium). All microcosms tested had depleted sulfate levels, including those with (phenol, *m*-cresol, *p*-cresol) and those without (control, *o*-cresol) stimulated methane production. The control microcosms were depleted as much as the other microcosms suggesting that the added phenolic substrates did not affect the extent of sulfate consumption but the data give no indication of the effects on rate of sulfate loss.

To determine the extent that sulfate is depleted in the samples during storage, two microcosms were prepared for samples MBC1-5 and MBC2-5 which contained 10 mL medium and 10 mL sample which had been stored at 4°C for 5 mon. The microcosms were shaken and allowed to sit for 1.5 h before subsamples were taken for sulfate analysis. The concentration of sulfate in these microcosms was 20 and 22 mg/L for MBC1-5 and MBC2-5, respectively. Considering the 1:1 inoculum to medium dilution, the samples MBC1-5 and MBC2-5 contained 40 and 44 mg/L sulfate, respectively after 5 mon storage at 4°C. At the time of collection in July 1998, the 5 m samples contained ≈87 mg/L sulfate, therefore the 40 mg/L loss of sulfate during storage suggests that that SRB are active at 4°C and that

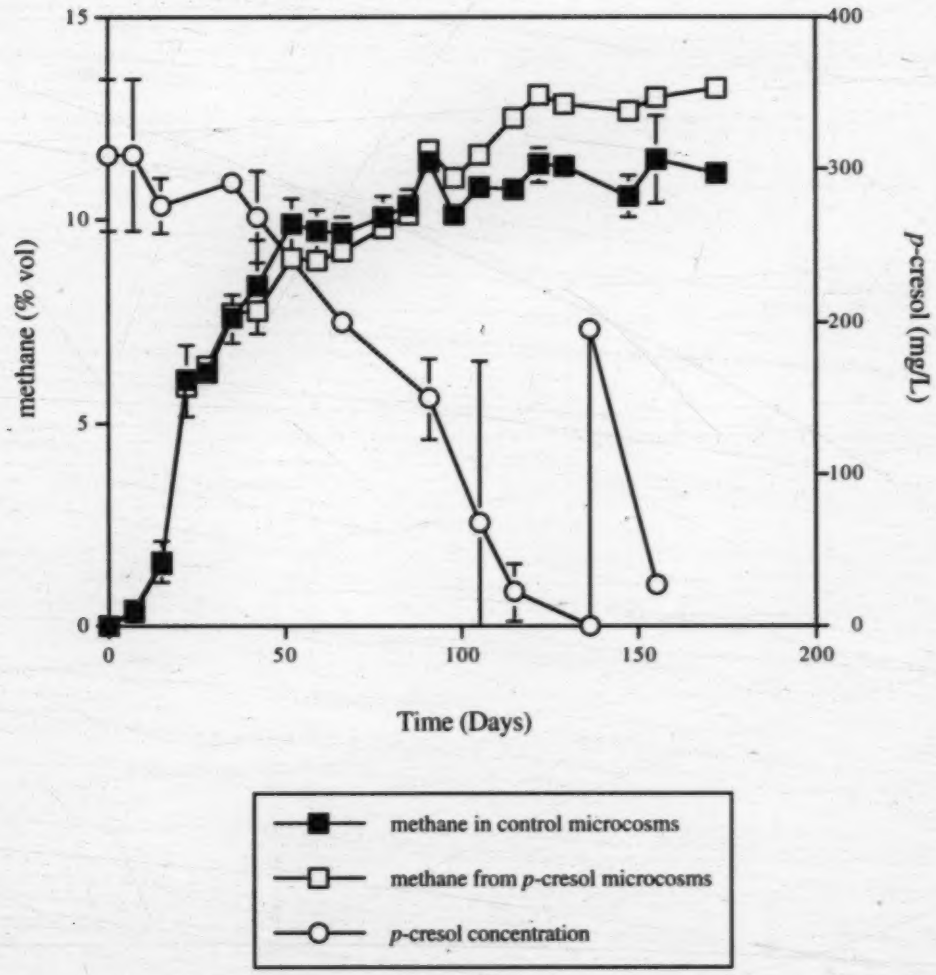


Figure 3.12: Methane production and loss of *p*-cresol in microcosms containing July 1998 sample MB2-5 and incubated at room temperature. The control received no *p*-cresol. Microcosms were re-supplemented on day 136. Each point represents the mean of triplicate microcosms. Bars for the *p*-cresol microcosms and controls represent one standard deviation.

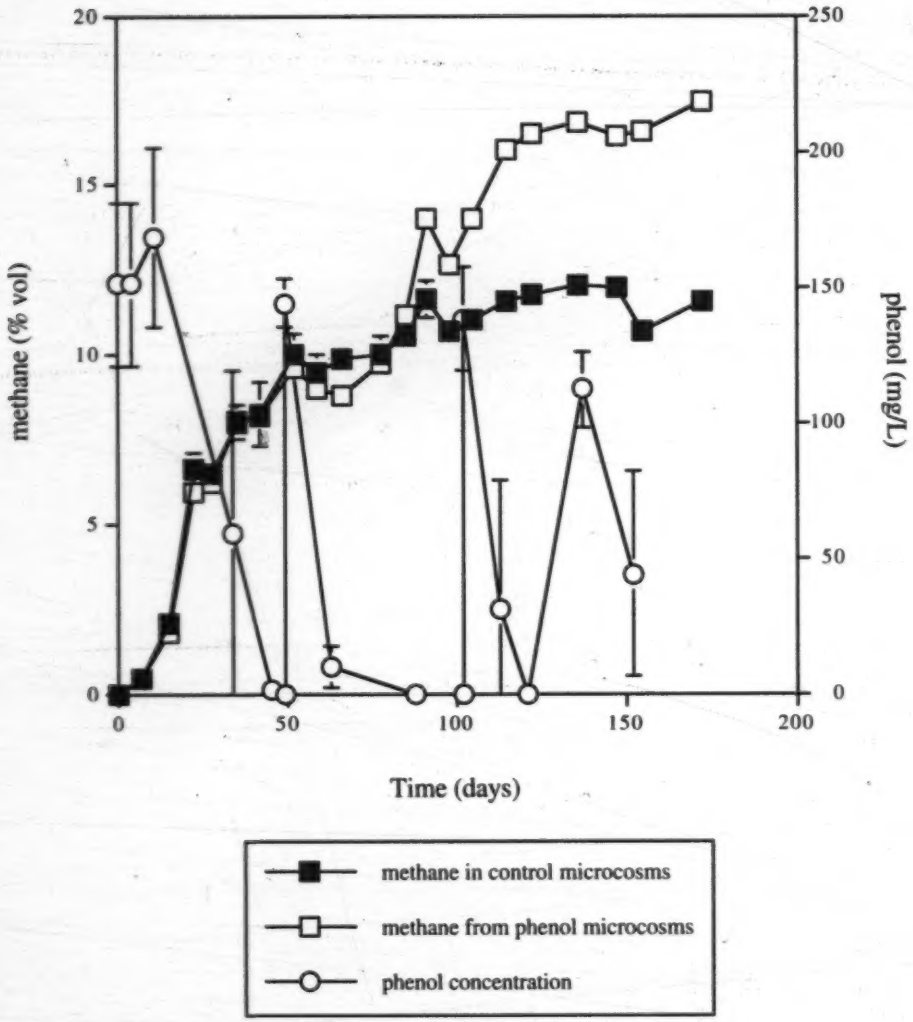


Figure 3.13: Methane production and loss of phenol in microcosms containing July 1998 sample MBC1-5 and incubated at room temperature. The control received no phenol. Microcosms were re-supplemented on days 49, 102 and 132. Each point represents the mean of triplicate microcosms. Bars for the phenol microcosms and controls (smaller than the symbol) represent one standard deviation.

some organics present in the sample are likely metabolized during storage. Consequently, not all of the sulfate loss in the microcosms can be attributed to phenol addition.

The overall loss of sulfate in the microcosms suggests that the SRB are actively metabolizing organic substrates within the fine tailings samples, but whether the SRB were responsible for the depletion of phenol without methane production cannot be conclusively determined. Other anaerobic bacteria involved in the hierarchy of substrate utilization may have been exerting influences that have not been quantified. The possibility of this is supported by the recognition that phenol consumption without methane production was noted in the MBWIP-10 microcosms, which had initial sulfate levels below 1.0 mg/L, that is, the SRB did not have a supply of inorganic TEAs. The high concentration of organic compounds in the fine tailings likely supports an active population of fermentative microorganisms. The fermenters may be responsible for the degradation of phenol to methanogenic substrates, which could include SRB that have switched to fermentative metabolism because of low sulfate concentrations (Postgate 1984).

The loss of substrate without methane production, as observed in the phenol-supplemented microcosms, occurred in the second experiment, which was inoculated with recently collected fine tailings samples (July 1998) but not in the experiment using aged samples (August 1997). The 1997 samples had depleted sulfate concentrations at the time of collection (<5 mg/L) and the experiment was not begun until after 8 mon of storage. Recognizing that metabolism continues, although slower, during storage at 4°C, it is conceivable that over the 8 mon storage, sulfate was depleted. When the phenol-supplemented microcosms were established, the methanogens would have had a competitive basis for obtaining the breakdown products from the added phenolic substrates, therefore methane production and substrate loss were concurrent events. If sulfate was initially depleted due to storage, in the first experiment, then the methanogens would not have had to overcome any substrate competition which may explain for why the first experiment had shorter lag times before stimulated methanogenesis than the second.

Regardless, the lag times before stimulated methane production were still longer than expected for substrates that are generally held to be amenable to utilization under methanogenic conditions (Fedorak and Hrudey 1984; Godsy et al. 1983; Wang et al. 1989). The prolonged delay could be indicative of the anaerobic consortium having to adapt to the presence of a new and novel substrate, or that there is a delayed degradation of the phenolic compounds to acetate by the consortium due to the presence of more readily utilizable substrates in the inoculum. Acetate stimulated methanogenesis within 15 to 28 d of incubation (Table 3.20, Figure 3.11) but phenol did not stimulate methanogenesis until after methane production in the controls had plateaued near 10% vol (Figure 3.11).

The presence of more readily utilizable substrates in the inoculum would also explain why none of the microcosms incubated at 14°C exhibited stimulated methane production when supplemented with phenols. If more easily oxidizable substrates are utilized prior to degradation of the phenols, then incubation at the lower temperature would prolong the time before utilization of the phenols, as anaerobic metabolism slows down at lower temperatures (Zeikus and Winfrey 1976). Evidence of slower or delayed metabolism at 14°C was noted in the acetate-supplemented microcosms incubated at 14°C, which took twice as long to utilized acetate as microcosms incubated at room temperature (Figure 3.11). Since acetate took almost 60 d to stimulate methanogenesis when stored at 14°C and only 15 to 28 d at room temperature, then it is likely that not enough time was given to observe degradation of the phenols and that the more easily utilized substrates have not been depleted enough to force the degradation of the phenols at 14°C.

The fine tailings samples have been shown to possess the ability to degrade phenol, *m*-cresol and *p*-cresol. However, degradation in microcosms was delayed for a long time and did not occur at an environmental temperature. There is little possibility these compounds are the major substrates supporting methanogenesis within the fine tailings which is consistent with the low concentrations of phenols in the tailings pond (Hargesheimer and Coutts 1984).

3.5.3 Naphthenic acids

NAs are released during extraction of bitumen from the oil sands and are concentrated as the water is recycled through the process. In the tailings pond, NAs concentrations range between 80 and 120 mg/L. Due to their abundance and successful degradation under aerobic conditions (Herman et al. 1994), NAs were considered likely candidates for methanogenic substrates. Studies with anaerobic sewage digestors have shown that long chain carboxylic acids undergo beta-oxidation to yield acetate for methanogens (Jeris and McCarty 1965; Chynoweth and Mah 1971). Similarly, it was thought that the carboxylated side chains of the NAs would undergo beta-oxidation to provide acetate to the methanogens leaving the cyclic portion untouched (CEATAG 1998).

In order to evaluate the ability of NAs to stimulate methanogenesis, three types of NAs were used: extracted, commercial and surrogate NAs. NAs were extracted from the surface waters of the MLSB to obtain a natural source and a commercial mixture was obtained from Kodak. Individual NAs are not commercially available, however, surrogates, or compounds with similar structure containing a ring with an aliphatic side chain are. Five surrogates tested included aliphatic compounds: 3-cyclohexylpropanoic acid, 4-cyclohexylbutanoic acid, 5-cyclohexylpentanoic acid and the aromatic compound, 6-phenylhexanoic acid (Figure 2.5). A study was also performed using a ^{14}C-labelled bicyclic NA (Figure 2.6).

3.5.3.1 Potential for methane biogenesis from commercial NAs in sewage sludge

After 480 d incubation at 37°C, none of the microcosms containing commercial NAs and sewage sludge demonstrated an increase in methane production over the controls (Figure 3.14) indicating that the commercial mixture of NAs does not support methanogenesis in sewage sludge. Microcosms containing 500, 1000 and 2000 mg/L commercial NAs had reduced methane production with greater inhibition noted in the 1000 and 2000 mg/L commercial NAs treatments. After 121 d the microcosms containing 1000

mg/L commercial NAs had an increase in methane production. No increase was noted in the microcosms containing 2000 mg/L commercial NAs. The effect of commercial NAs on methane production may be hindering the methanogens directly, or disrupting other microorganisms which provide substrates to the methanogens. The slight recovery in methane production in the microcosms containing 1000 mg/L NAs suggests that tolerance has developed and that the affected population(s) have overcome the toxicity, enabling methanogenesis to resume. The plateau of methane production in these microcosms below control values is likely a result of depleted concentrations of available substrates. That the microcosms containing 2000 mg/L commercial NAs did not recover suggests that the toxicity is too stringent to overcome. Mixtures of NAs are commonly used as an effective fungicide for preventing wood decay (St. John et al. 1998), therefore the toxicity against the microbial consortium in the sewage sludge is not surprising.

This was one of the first experiments performed and was initiated before the fine tailings samples in August 1997 were collected. As such, sewage sludge was used as an inoculum and the microcosms were incubated at 37°C, a temperature suitable for this sample. The preliminary results obtained from the sewage sludge provided focus in the designing of later experiments. This was a valid endeavor because sewage sludge is one of the best studied and most easily manipulated methanogenic samples and is representative of other habitats in which organic matter is completely degraded to methane (Zinder 1993).

3.5.3.2 Potential for methane production from NAs extracted from MLSB

NAs extracted from MLSB were added to microcosms in concentrations ranging from 50 to 500 mg/L above the *in situ* concentrations. Eight August 1997 samples (MB1-5, MB1-15, MB1-20, MB2-5, MB2-15, MB2-20, MBIP1-5 and DP-5) were tested (Table 3.21). All the samples had the same trends and so MB1-20 is provided as a representative sample (Figure 3.15A). Inhibition of methanogenesis was determined by comparing methane concentrations using Dunnett's statistical method. Methane production recovered

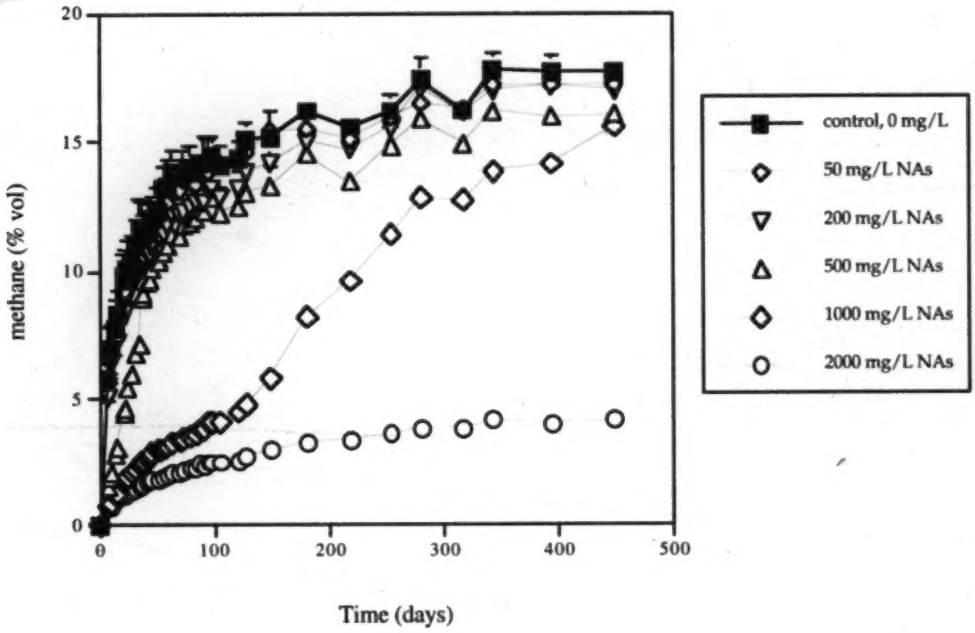


Figure 3.14: The effect of commercial NAs on methanogenesis in microcosms inoculated with sewage sludge. Each point represents the mean of triplicate microcosms and bars for the control represent one standard deviation.

from inhibition when methane concentrations in the treatment microcosms reached the same levels as the control microcosms. In these situations, because inhibition of methanogenesis was overcome with further incubation, methanogenesis was classified as being delayed.

Extracted NAs added to a concentration of 50 mg/L had no effect on methanogenesis (data not shown) while 150 mg/L extracted NAs caused a slight delay in methanogenesis which was overcome eventually (Table 3.21 and Figure 3.15A). Addition of 300 mg/L caused an initial delay in methane production which, with prolonged incubation, recovered and produced methane concentrations equal to the controls. The length of time required before the microcosms supplemented with 300 mg/L extracted NAs recovered differed for each of the samples tested ranging from 55 to 150 d. There was no direct relationship noted between time of recovery and depth or location of sample or the abundance of methanogens present. Microcosms supplemented with 500 mg/L extracted NAs were inhibited more than the microcosms supplemented with 300 mg/L extracted NAs. After prolonged incubation, methane values began to rise in these microcosms but none produced methane values close to those of the control microcosms (Table 3.21). MBIP1-5 which has the largest methanogen population(MPN value $>10^7$/g) was the sample least affected by the addition of 300 and 500 mg/L extracted NAs. The addition of acetate at 680 mg/L stimulated methane production in all microcosms between 27 and 48 d.

There was no evidence that the NAs extracted from the surface water of MLSB could directly support methanogenesis under the experimental conditions used. Extracted NAs either had no effect, or delayed or inhibited methanogenesis. Inhibition only occurred in those microcosms containing NAs in concentrations (150, 300 and 500 mg/L) which were greater than the *in situ* concentration of NAs in the surface water suggesting that the bacterial communities have adapted to the basal level of NAs (80 to 120 mg/L) in the MLSB. Recovery of methanogenesis was observed in a majority of the microcosms inhibited by the addition of NAs, thus, it appears that the bacterial populations acclimate to the exposure to the exposure of higher concentrations of NAs. Adaptation in the microbial

Table 3.21: Summary of the effects of increasing concentrations of extracted NAs on methanogenesis in August 1997 fine tailings samples incubated at room temperature. Reported are the days at which inhibition of methanogenesis was statistically significant (inhibition column) and when methane production returned to the control values (recovery column). Final CH_4 (% vol) was measured on day 231.

	control	150 mg/L		300 mg/L		500 mg/L	
Sample	final CH_4 (% vol)	inhibition (day)	recovery (day)	inhibition (day)	recovery (day)	inhibition (day)	final CH_4 (% vol)
MB1-5	10	27	69	5	160	5	1.8
MB1-15	14	NI[b]	NR[c]	20	55	5	11
MB1-20	10	NI	NR	20	141	20	2.7
MB2-5	11	20	69	5	141	5	5.3
MB2-15	11	20	69	5	100	5	8.3
MB2-20	11	20	83	20	100	5	7.9
MBIP1-5	12	20	48	5	55	5	8.9
DP-5[a]	10	20	55	20	160	20	5.8

[a]one replicate of the controls which skewed statistical analysis
[b]no statistically significant inhibition
[c]no recovery observed as there was no initial inhibition

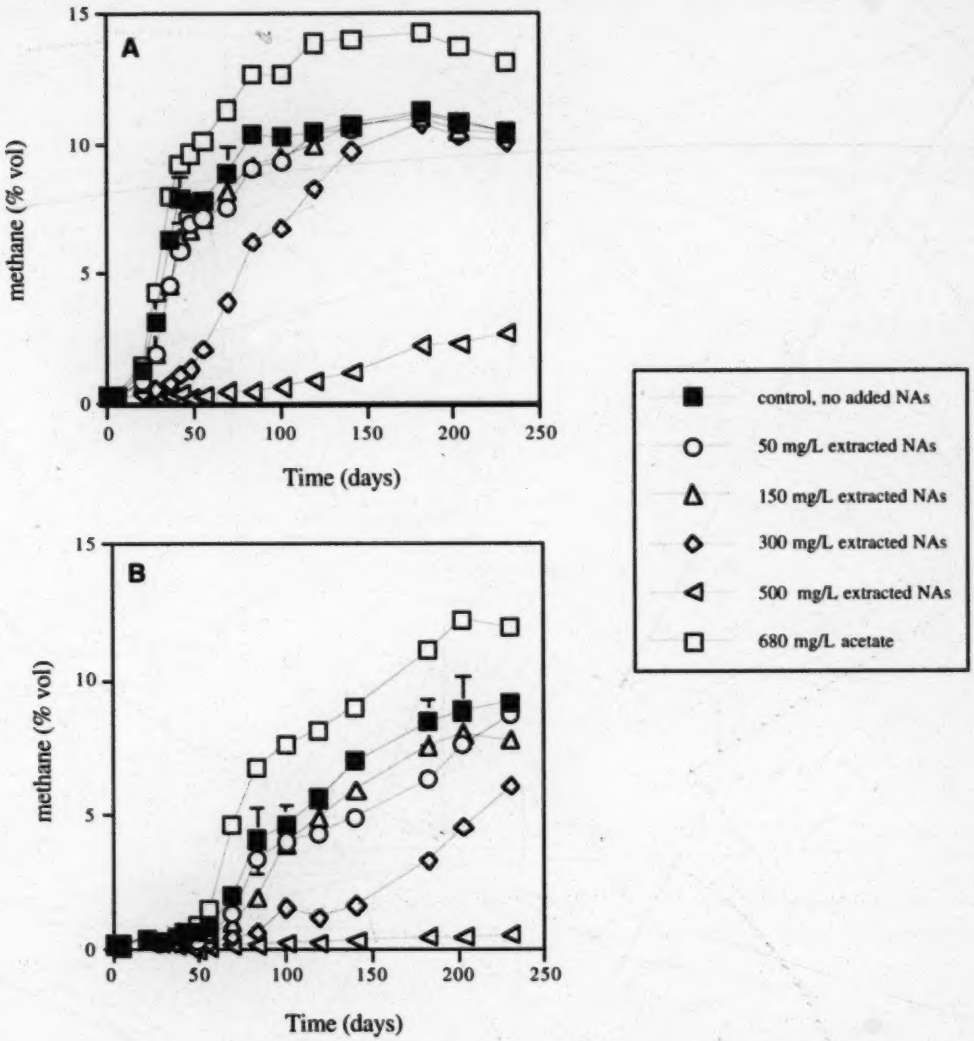


Figure 3.15: The effect of extracted NAs on methanogenesis in August 1997 sample MB1-20 incubated at room temperature (A) and 14°C (B). Points are the mean of triplicate microcosms and bars for the control represent one standard deviation. The *in situ* concentration of NAs was 88 mg/L (Table 3.1).

consortium could create greater tolerance to NAs or the compounds could be degraded to non-toxic levels enabling methanogenesis to proceed. Although the addition of extracted NAs did not stimulate methanogenesis, it is possible that the consortium can degrade NAs to non-methanogenic substrates or that the microcosm study did not mimic *in situ* conditions closely enough to show the contribution of methanogenic substrates from extracted NAs.

Microcosms inoculated with sample MB1-20 were incubated at room temperature (Figure 3.15A) and at 14°C (Figure 3.15B). Incubation at the different temperatures produced the same trends. The only difference noted was that the microcosms incubated at 14°C did not have methanogenic activity until after day 50, whereas those incubated at room temperature were active by day 25. In general, enzyme and total metabolic activity increases by a factor of two, with every 10°C rise in temperature (Atlas and Bartha 1993).

The fine tailings samples produce high background concentrations of methane and if only a portion of the NAs were supporting methanogenesis and only a small increase in methane occurred, it would not necessarily be observed over the high background or could be diluted in the 105-mL headspace of the microcosms. These microcosm studies may not have been sensitive enough to detect stimulated methanogenesis if only a small portion of the compounds in the NA mixture was susceptible to anaerobic biodegradation. Biodegradation of specific compounds in a complex mixture can be detected if the mixture is analyzed before and after exposure to an active microbial culture. For example, Fedorak and Westlake (1983) demonstrated the selective removal of biphenyl and some methylbiphenyl from the aromatic fraction of Prudhoe Bay crude oil by monitoring the loss of the specific compounds. A suitable technique for the analysis of individual compounds within the NA mixture is not available. However a new method developed by St. John et al. (1998) using gas chromatography-electron impact mass spectrometry (GC-EIMS) can be used to determine the percent composition of specific NA components. Making *tert*-butyldimethylsilyl derivatives of the NAs creates a relatively stable structure that has less

fragmentation then other derivatives when exposed to GC-EIMS. Since the *tert* derivative does not fragment extensively, GC-EIMS analysis yields both molecular mass and structural information. This information can be used to classify the individual components eluting within the usually large, unresolved NA peak area, providing percent composition of NA components based on Z and carbon numbers. The GC-EIMS method is a simple method, using commonly available instrumentation, which could be used to monitor the changes in the composition of the NAs mixture in microcosm studies.

Other methods using more sophisticated instruments such as Fast Atom Bombardment Mass Spectrometry (FABMS) and Electrospray Ionization Mass Spectrometry (ESIMS) are also available. Both of these methods have been used to study NAs (Morales et al. 1993; Morales-Izquierdo 1999). FABMS uses an atom beam to bombard a non-volatile liquid sample. The molecules are ionized and are detected by mass spectrometric analysis. FABMS is a powerful qualitative analysis but is not ideal for quantitative work with a complex mixture (Morales et al. 1993). The FABMS would not be useful for monitoring the loss of components of a NAs mixture.

ESIMS does not artificially produce ions but uses the charged nature of the analyte molecules in solution for detection. A fine spray of charged droplets is produced which undergoes ion evaporation in an applied electrical field and the ions are analyzed. The power of this technique is that it detects intact species and offers reproducibility needed for quantitative analysis (Morales-Izquierdo 1999). The ESIMS method is significantly more involved and requires more specialized instrumentation than the GC-EIMS method.

3.5.3.3 Toxicity testing of the direct inhibition of methanogens by NAs

Having noted in previous experiments (sections 3.5.3.1 and 3.5.3.2), that extracted and commercial NAs inhibited methane production, the next question was whether the addition of NAs was directly toxic to the methanogens or to the microorganisms that provided methanogenic substrates. Commercial and extracted NAs were tested in concentrations of 50, 90 and 150 mg/L. Each of the microcosms established (except for the

negative controls) received acetate or H_2 as an additional substrate for the methanogens. If methane production was inhibited, then the methanogenic population was being directly affected because the presence of acetate or H_2 removed the reliance of the methanogens on other members of the anaerobic consortium to provide suitable substrates. If methane production remained unaffected, then the NAs were likely toxic to other members of the consortium.

Extracted and commercial NAs were tested on August 1997 samples MB1-5, MB2-5, MB1-20, MB2-20, DP-5 and MBIP1-5. The results from each of these sets of microcosms were essentially identical so for simplicity, only the results for MB1-20 have been reported (Figures 3.16 to 3.18).

Addition of H_2 stimulated methanogenesis in the microcosms (Figure 3.16) whereas supplementation with 340 mg/L acetate did not (Figure 3.17). Addition of the commercial NAs at concentrations of 50 or 90 mg/L had no observable effect on methane production whereas addition of 150 mg/L NAs caused initial inhibition between day 9 and 15. This inhibition was overcome as methane production, reached control levels between day 34 to 65 (Figures 3.16B and 3.17B). The addition of extracted NAs at a concentration of 50 mg/L had no observable effect. Higher concentrations (90 and 150 mg/L) of extracted NAs caused initial inhibition between day 4 to 29, which was overcome as methane production reached control levels between day 34 to 107 (Figures 3.16A and 3.17A). The one exception noted was microcosms containing sample MB2-5, acetate and 90 mg/L extracted NAs which had stimulated methane production over controls (data not shown). This slight stimulation was only noted in one of the six samples tested.

Microcosms containing MB1-20 and acetate or H_2 were incubated at room temperature and 14°C (data not shown). The trends between the two different incubation conditions were the same, with activity slower in the microcosms incubated at 14°C. The time before initial methane production was 25 and 50 d, respectively, for the microcosms

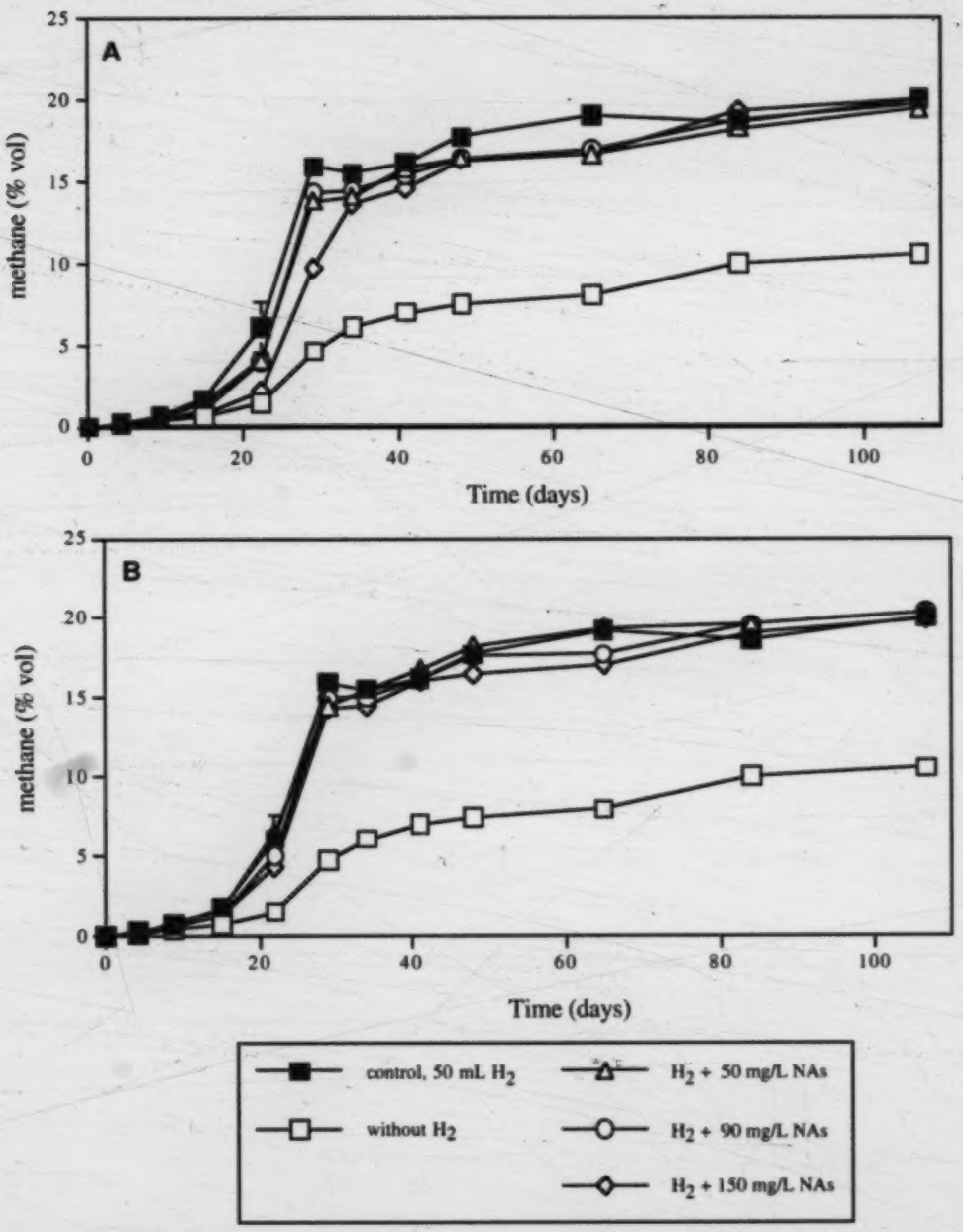


Figure 3.16: The effect of extracted (A) and commercial (B) NAs on methanogenesis in H_2-supplemented microcosms containing August 1997 sample MB1-20 and incubated at room temperature. Each point represents the mean of triplicate microcosms and the bars for the controls represent one standard deviation.

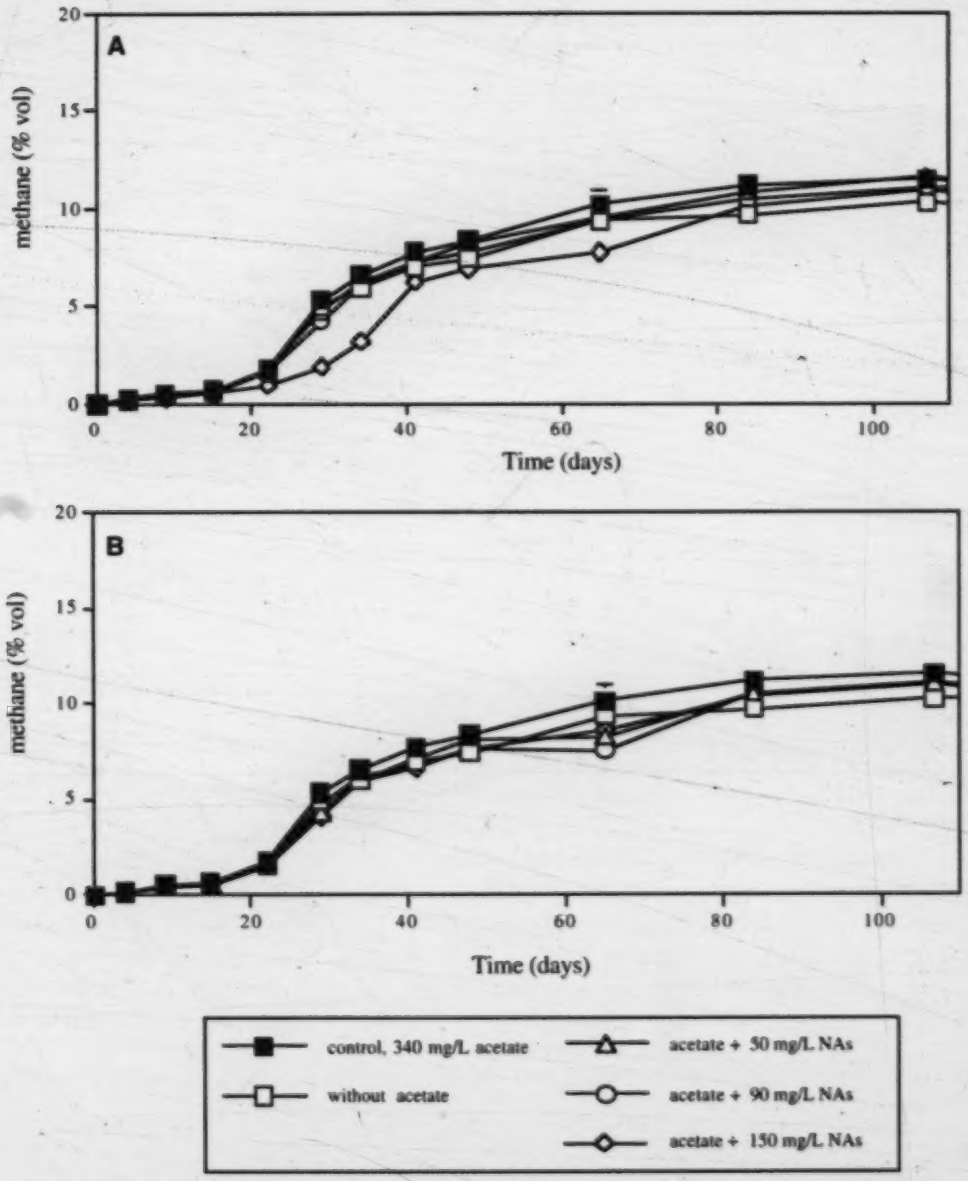


Figure 3.17: The effect of extracted (A) and commercial (B) NAs on methanogenesis in acetate-supplemented microcosms containing August 1997 sample MB1-20 and incubated at room temperature. Each point represents the mean of triplicate microcosms and the bars for the controls represent one standard deviation.

incubated at room temperature and 14°C. The difference in time was expected as the rate of methanogenesis is slower at low temperatures (Conrad et al. 1989; Schink 1997).

The addition of H_2 stimulated methanogenesis by the consortium, and the presence of NAs (extracted and commercial) in concentrations >90 mg/L delayed methanogenesis when usable substrate was present. Over the short term, the H_2-utilizing methanogens were directly affected. Because methane concentrations in the NA-supplemented microcosmseventually reached the same levels as the controls, the H_2-utilizing methanogens can overcome this toxicity or NAs concentrations drop to non-toxic levels.

Since acetate concentrations of 680 mg/L stimulated methanogenesis in August 1997 samples previously (section 3.5.3.2, Figure 3.15), it was speculated that the 340 mg/L acetate originally added to the microcosms was insufficient to stimulate methanogenesis to a detectable level (Figure 3.17). Establishing the successful stimulation of methanogenesis by acetate was required to determine whether the NAs were exhibiting direct inhibitory effects on the acetate-utilizing methanogens. All the acetate-containing microcosms were re-supplemented with an additional 900 mg/L acetate on day 120 and all produced significantly more methane than the controls without acetate (Figure 3.18). Since the microbial populations had already adapted to the effects of the NAs as the methane concentrations in the acetate-supplemented controls and NAs-supplemented microcosms were the same by day 107 (Figure 3.17), addition of more acetate on day 120 (Figure 3.18) did not clarify whether the methanogens were being directly affected by the NAs. Methanogenesis in the microcosms re-supplemented with 900 mg/L acetate was stimulated immediately and produced the same amount of methane in each set of microcosms regardless of the concentration of NA added (Figure 3.18). Therefore, if the NAs had been directly affecting the acetate-utilizing methanogens, the toxicity was overcome with time and did not hamper acetate-utilization.

A previous experiment (section 3.5.3.2) using extracted NAs found that 300 and 500 mg/L NAs significantly inhibited methanogenesis. In the present experiment, extracted

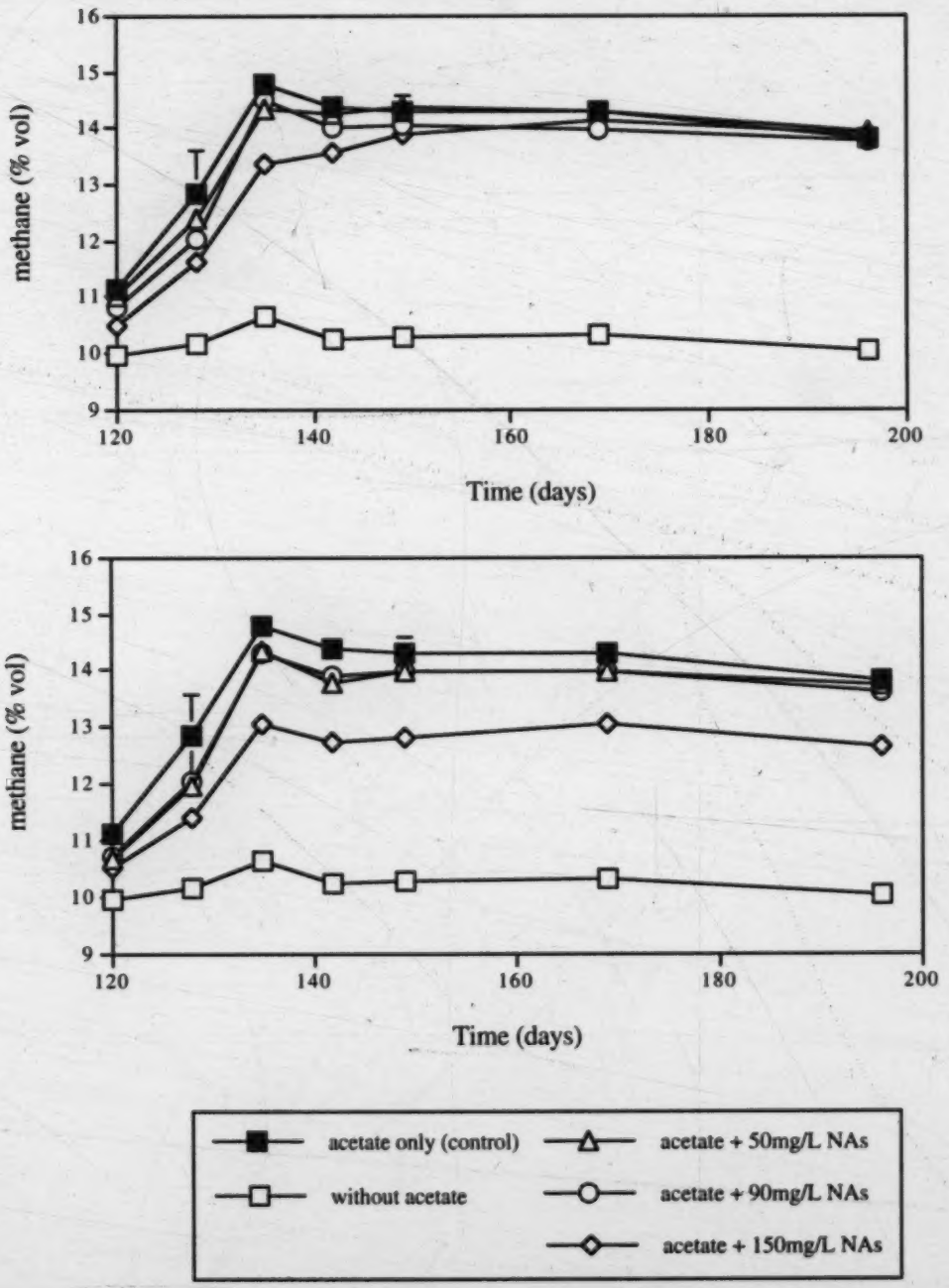


Figure 3.18: The effect of re-supplementation with 900 mg/L acetate on day 120 on methanogenesis in acetate-supplemented microcosms containing August 1997 MB1-20 and extracted (A) and commercial (B) NAs and incubated at room temperature. Each point represents the mean of triplicate microcosms and the error bars for the controls represent one standard deviation.

NAs (>90 mg/L) were toxic to the H_2-utilizing methanogens and possibly toxic to the acetate-utilizing methanogens. It is likely, but not confirmed that inhibition by 300 and 500 mg/L extracted NAs was due to direct toxicity to the methanogenic populations.

The exposure of the fine tailings samples to NAs extracted from MLSB resulted in more cases of delayed methanogenesis than microcosms which received commercial NAs. Of the 27 sets of triplicate microcosms which received extracted NAs, 18 had some level ofinhibited methanogenesis, where as only 9 sets of microcosms which received commercial NAs had delayed methanogenesis. One would hypothesize that because the consortium is exposed *in situ* to the NAs extracted from the MLSB that addition of the same compounds would not have had such an adverse effect.

The commercial mixture (Kodak NAs sodium salts) has been characterized by Morales (1999) using ESIMS and was shown to contain mostly NAs belonging to the Z=0, -2, and -4 groups (linear, one- and two-ringed compounds). Similar analysis of the *in situ* NAs from Syncrude Canada Ltd. tailings pond water showed a shift in NAs groups to the higher Z and carbon numbers with equal proportions of Z groups 0, -2, -4, -6 and -8 (linear to four-ringed structures). The commercial mixture has a potentially less toxic composition because of the dominance of linear, one- and two-ringed structures than the natural NAs which has compounds with more rings. The commercial mixture likely lacks the individual NA compounds found in the MLSB, which are toxic to the methanogens.

3.5.3.4 Potential for methane biogenesis from surrogate NAs

3.5.3.4.1 In sewage sludge

The surrogate NAs chosen were: 3-cyclohexylpropanoic acid, 4-cyclohexylbutanoic acid, 5-cyclohexylpentanoic acid, and 6-phenylhexanoic acid. The effects of surrogate NAs on methanogenesis was tested on sewage sludge three times. The first experiment (described in section 2.5.3.4.1) was incubated for 223 d at 37°C, the second experiment (described in section 2.5.3.4.2) was incubated for 228 d at room temperature and the third

experiment (described in section 2.5.3.4.3) was incubated for 132 d at room temperature. The results of the first two experiments are presented together (Table 3.22).

In the first two experiments, hexadecanoic acid served as a positive control and resulted in the rapid stimulation of methanogenesis (Figure 3.19). Increasing the concentration of hexadecanoic acid produced greater volumes of methane (Table 3.23).

In experiment one, addition of 3-cyclohexylpropanoic acid when present at 400, 600 and 800 mg/L stimulated methanogenesis by days 22, 39 and 118 respectively. Microcosms containing 400 and 600 mg/L 3-cyclohexylpropanoic acid were re-supplemented which stimulated methanogenesis further. Similarly, in experiment two, 400 and 600 mg/L 3-cyclohexylpropanoic acid enhanced methanogenesis, producing 14 and 35% of the expected methane (Table 3.23). Re-supplementation caused further stimulation and on the last day of analysis (177 d), 57 and 25% of the expected amount of methane had been produced by the microcosms containing 400 and 600 mg/L 3-cyclohexylpropanoic acid, respectively. Methane concentrations at 177 d had not plateaued so it is likely that more methane would have been produced by these microcosms with further incubation.

In experiments one and two, 4-cyclohexylbutanoic acid failed to stimulate methanogenesis. Concentrations of 400 and 600 mg/L caused a slight delay in methanogenesis in the first experiment but this was overcome with prolonged incubation.

The addition of 5-cyclohexylpentanoic acid had various effects on methanogenesis. In experiments one and two, 200 mg/L 5-cyclohexylpentanoic acid stimulated methanogenesis, which was repeated after re-supplementation. In the second experiment, 150% of the expected amount of methane was generated (Table 3.23), recovery of more than 100% of the predicted methane is likely due to addition of more than 200 mg/L 5-cyclohexylpentanoic acid to the microcosms which was the concentration used to calculate the predicted volume of methane. Re-supplementation further stimulated methane production and on the last day of analysis (177 d) 20% of the expected methane had been

produced, however methane levels had not plateaued and it is likely that more methane would have been produced in these microcosms.

6-Phenylhexanoic acid was only tested in experiment one. It stimulated methanogenesis after 39 days at 200 and 400 mg/L concentrations. Re-supplementation resulted in further methane production.

Hexadecanoic acid was the compound best able to stimulate methane production as it is a straight-chain fatty acid most susceptible to beta-oxidation by a consortium. It is speculated that degradation of NAs begins with beta-oxidation of the aliphatic side chain (CEATAG 1998) and methane production would be stimulated the most by 6-phenylhexanoic acid, as it has the longest side chain and forms intermediates which are subject to further degradation to methane. 6-Phenylhexanoic acid would be followed by 5-cyclohexylpentanoic acid, 4-cyclohexylbutanoic acid, 3-cyclohexylpropanoic acid, which may not form degradable intermediates and are ordered by decreasing number of carbons in their side chains. There was little correlation between methane production and the concentration of a given substrate, that is, production of methane did not increase with increasing concentrations of surrogate NAs. Nor did methane produced correlate with the complexity of a given surrogate, that is, methane production did not increase with increasing length of the side chain (Table 3.23). Nor did methane production consistently decrease with increasing complexity of the surrogate NA added. Therefore, the degradability of surrogate NAs is dependent on the type, structure and concentration of a given NA. Based on the slight discrepancies between experiments 1 and 2, degradability of the surrogate NAs is also inoculum dependent.

A third experiment studying surrogate NAs and sewage sludge was designed using serum bottles (158-mL) inoculated with 50 mL sewage sludge, 50 mL medium containing and 3-cyclohexylpropanoic acid (200, 400, 600, or 800 mg/L) or 200 mg/L 5-cyclohexylpentanoic acid. These surrogate NAs were chosen because they had previously shown to be degraded by sewage sludge. These microcosms were established to generate

Table 3.22: Summary of the effects of the addition of different concentrations of surrogate NAs on the production of methane in microcosms inoculated with sewage sludge. Microcosms in experiment 1 were incubated at 37°C for 223 d and those in experiment 2 were incubated at room temperature for 228 d.

Compound	200 mg/L	400 mg/L	600 mg/L	800 mg/L
Experiment 1				
3-cyclohexylpropanoic acid	no[a]	+[b]	+	+
4-cyclohexylbutanoic acid	no	-/no[c]	-/no	no
5-cyclohexylpentanoic acid	+	no	-[d]	no
6-phenylhexanoic acid	+	+	no	no
Experiment 2				
3-cyclohexylpropanoic acid	no	+	+	NT[e]
4-cyclohexylbutanoic acid	no	no	no	NT
5-cyclohexylpentanoic acid	+	no	-/no	NT
6-phenylhexanoic acid	NT	NT	NT	NT

[a]no statistical difference between treatments and the controls
[b]statistically significant increase in methane production of methane over the control
[c]initial inhibition with a recovery in methane production to control levels
[d]statistically significant inhibition in methane production relative to the control
[e]condition was not tested

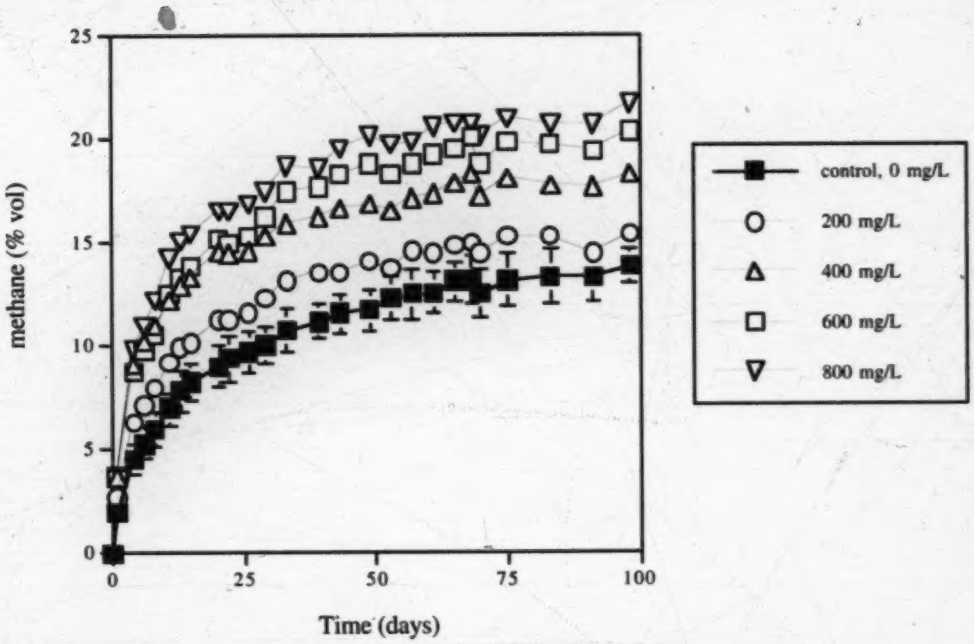


Figure 3.19: The effect of hexadecanoic acid on methanogenesis in microcosms containing sewage sludge. Each point represents the mean of triplicate microcosms and bars for the controls represent one standard deviation.

Table 3.23: Summary of the volume of CH_4 produced at STP from surrogate NAs in microcosms containing sewage sludge. Data are from experiment 2. Expected volumes of methane were calculated using Buswell's equation and assume mineralization of the whole compound.

Substrate	dry CH_4 produced (mL)	expected dry CH_4 (mL)	% of expected CH_4
400 mg/L 3-cyclohexylpropanoic acid	1.45	10.3	14
600 mg/L 3-cyclohexylpropanoic acid	5.37	15.5	35
200 mg/L 5-cyclohexylpentanoic acid	8.20	5.48	150
200 mg/L hexadecanoic acid	10.0	6.04	166
400 mg/L hexadecanoic acid	11.9	12.1	98
600 mg/L hexadecanoic acid	10.4	18.1	57

inoculum enriched for degradation of the surrogate NAs. The enriched inoculum was to be used to prepare new microcosms able to actively metabolize the surrogate NAs without the long acclimatization and lag times noted in the previous experiments. The purpose of these "enriched" microcosms was to study the potential metabolites produced during degradation of the surrogate NAs. Due to time constraints the metabolite study was not initiated.

Both 3-cyclohexylpropanoic (Figure 3.20) and 5-cyclohexylpentanoic acid (data not shown) stimulated methanogenesis in the sewage sludge microcosms. The microcosms produced over 90% of the expected amount of methane from the added surrogates. On day 79 these microcosms were re-supplemented which further stimulated methanogenesis and on day 132 the microcosms were analyzed for the last time and had produced over 80% of the expected methane in each of the microcosms supplemented with 3-cyclohexylpropanoic acid and 32% of the expected methane from 200 mg/L 5-cyclohexylpentanoic acid. Methanogenesis had not plateaued by this analysis point, so it is expected that more methane would have been produced from the microcosms.

It is apparent that sewage sludge can degrade 3-cyclohexylpropanoic acid and 5-cyclohexylpentanoic acid. Methane yields were high and generally close to the amount of methane predicted by Buswell's equation for the complete mineralization of the compounds. It is unlikely that only the carboxylic side chains are being degraded by beta-oxidation, but that the ring structure is being broken. Metabolite studies would provide further evidence for ring cleavage and the possible mechanisms involved. There are no previous reports in the literature of these two compounds being degraded under anaerobic conditions.

3.5.3.4.2 In fine tailings

Having established that some surrogate NAs can be degraded under methanogenic conditions with sewage sludge, microcosms were prepared using July 1998 fine tailings samples, MBC1-8, MBC2-15 and MBWIP-10. In this study, 3-cylcohexylpropanoic acid,

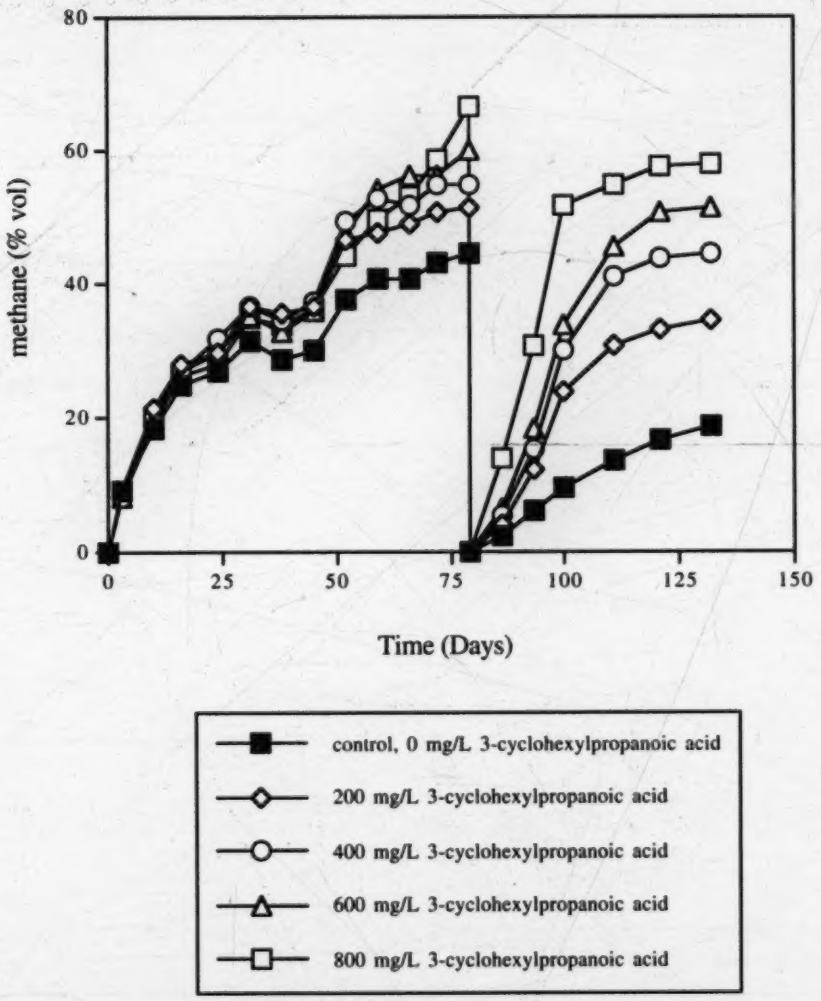


Figure 3.20: The effect of 200, 400, 600 and 800 mg/L 3-cyclohexylpropanoic acid on methanogenesis in microcosms inoculated with sewage sludge and incubated at room temperature. On day 79, the microcosms were flushed to remove a majority of the methane and were re-supplemented with 3-cyclohexylpropanoic acid. Each point represents the mean of triplicate microcosms and the bars for the control are smaller than the symbol and represent one standard deviation.

4-cyclohxylbutanoic acid and 5-cyclohexylpentanoic acid were tested in 200, 400 and 600 mg/L concentrations. Hexadecanoic acid and acetate served as positive controls.

Hexadecanoic acid stimulated methane production in the microcosms containing fine tailings samples, however the lag time prior to stimulation of methanogenesis (20 to 48 d) was longer than for the sewage sludge microcosms (5 d). Acetate-induced methanogenesis began after 19 d and resulted in the production of 60 to 120% of the expected methane (data not shown). The concentration of acetate initially added to the microcosms may have been higher than the 500 mg/L concentration used to calculate the value for % recovery, therefore more than 100% of the predicted methane was produced in some of the microcosms.

Methanogenesis was inhibited by the surrogate NAs microcosms containing fine tailings samples. Twenty-five of the twenty-seven sets of microcosms were inhibited at some point in the experiment and only six sets recovered to control levels after prolonged incubation (Table 3.24). The only two sets of microcosms which did not have methanogenesis inhibited contained sample MBWIP-10, supplemented with 200 mg/L 3-cyclohexylpropanoic acid and 200 mg/L 4-cyclohexylbutanoic acid. These microcosms stimulated methanogenesis late in the experiment, (>175 d) and only after the methane production in the microcosms had already plateaued. This implies that the consortium within the fine tailings samples are preferentially using organics other than the surrogate NAs to support methanogenesis, but that upon depletion of these more readily utilizable substrates adaptations may allow the population to metabolize these NAs to methane. Although 200 mg/L 3-cyclohexylpropanoic acid and 200 mg/L 4-cyclohexylbutanoic acid stimulated methanogenesis, increasing concentrations (400 and 600 mg/L) and more complex compounds (5-cyclohexylpentanoic acid) inhibited methanogenesis, so although adaptation and utilization of some surrogate NAs is possible, the surrogate NAs appear to have toxic effects in concentrations higher than 200 mg/L.

Methane concentrations in the stimulated microcosms reached 91 and 70% of the expected methane to be produced from 200 mg/L 3-cyclohexylpropanoic acid and 200 mg/L 4-cyclohexylbutanoic acid, respectively. The headspace gas in these microcosms was flushed to reduce methane concentrations to zero and were re-supplemented. Addition of substrate further stimulated methane production (Figure 3.21) producing 88 and 74% of the expected amount of methane from 200 mg/L 3-cyclohexylpropanoic acid and 200 mg/L 4-cyclohexylbutanoic acid, respectively. The microcosms were re-supplemented again on day 113 and methane production continued to be stimulated.

Why sample MBWIP-10, collected from the Base Mine Lake was able to utilize selective surrogate NAs while the MLSB samples (MBC1-8 and MBC2-15) were not, is not known. If only the side chains of these compounds were degraded in the MLSB fine tailings samples an increase in methane concentrations may not have been detected. Beta-oxidation of the side chain would produce 0.75 mL methane from 200 mg/L 3-cyclohexylpropanoic acid and 0.69 mL from 200 mg/L 4-cyclohexylbutanoic acid. In the MBWIP-10 microcosms over 5 mL methane was produced by the addition of the surrogate NAs, suggesting that not only the side chain was being degraded, but that ring cleavage may also be occurring. If ring cleavage was possible in MBWIP-10, then if the microorganisms responsible were present in the other samples, an increase in methane concentration in the microcosms would have also been observed. That methanogenesis was not stimulated in the other samples suggests that the microorganisms responsible for degrading the surrogate NAs were not present or they were not induced to metabolize the surrogates tested. But why the other samples failed to convert the surrogate NAs to methane is unknown. Perhaps transferring the fine tailings from the MLSB to the Base Mine Lake induces modifications in the consortium, changes which enable them to degrade the surrogate NAs.

The limited ability of the fine tailings samples to utilize the surrogate NAs supports the previous conclusion that degradation of the surrogate NAs is dependent on the type of

Table 3.24: Summary of the effects of the addition of different concentrations of surrogate NAs on the production of methane in microcosms inoculated with July 1998 samples MBC1-8, MBC2-15 and MBWIP-10. Microcosms were incubated at room temperature.

	MBC1-8			MBC2-15			MBWIP-10		
Concentration (mg/L)	200	400	600	200	400	600	200	400	600
3-cyclohexylpropanoic acid	-/no[a]	-[b]	-	-/no	-	-	+[c]	-/no	-
4-cyclohexylbutanoic acid	-/no	-	-	-	-	-	+	-	-
5-cyclohexylpentanoic acid	-/no	-/no	-	-	-	-	-	-	-

[a]initial inhibition with a recovery in methane production to control levels
[b]statistically significant inhibition in methane production relative to the control
[c]statistically significant increase in methane production of methane over the control

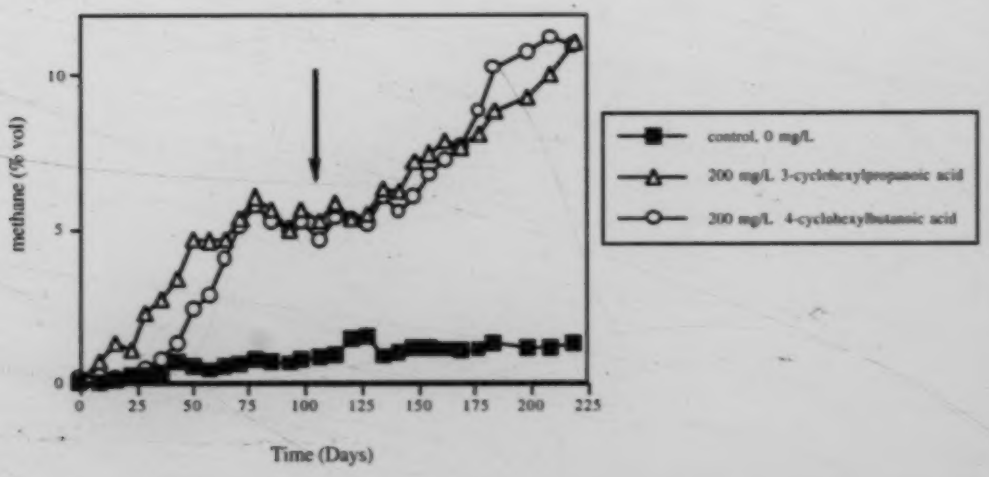


Figure 3.21: Stimulation of methanogenesis by 200 mg/L 3-cyclohexylpropanoic acid and 200 mg/L 4-cyclohexylbutanoic acid after re-supplementation in microcosms containing July 1998 sample MBWIP-10 and incubated at room temperature. After 183 d incubation from the start of the experiment, the microcosms were flushed and re-supplemented (day 0). The microcosms were re-supplementated on day 113 (denoted by arrow). Each point represents the mean of triplicate microcosms and bars for the controls represent one standard deviation and are smaller than the symbol.

surrogate, the concentration and the inoculum used. Fine tailings samples from MLSB demonstrated no ability to degrade the surrogate NAs under the conditions tested. The limited success of the Base Mine Lake sample (MBWIP-10) needs to be confirmed but may suggest that the potential for the degradation of NAs to methane exists.

3.5.3.5 Potential for anaerobic mineralization of a ^{14}C-DHNA

Pinto et al. (1998) recently reported the successful degradation of a surrogate NA of the Z=-4 family under aerobic conditions using wetland sediments obtained from the Suncor Inc. lease. With successful mineralization under aerobic conditions, the next question was whether degradation was possible under methanogenic conditions.

After 252 d incubation, the bicyclic surrogate NA, decahydro-2-naphthoic acid-8-^{14}C (DHNA) was not mineralized to $^{14}CO_2$ and/or $^{14}CH_4$. Activity was detected in the positive controls containing ^{14}C-acetate on the first day of analysis (24 d). 0.86 and 13.5% of the original activity added has been detected as $^{14}CO_2$ and $^{14}CH_4$, respectively in microcosms inoculated with MBWIP-10. $^{14}CO_2$ and $^{14}CH_4$ account for 0.54 and 36% of the original activity added to the positive controls containing sample MBC2-15. The positive controls confirm that experimental procedures were successful and that the detection system was functional. If the consortium was able to mineralize DHNA, the release of the label as CO_2 and CH_4 would be apparent. Consequently, it is unlikely that the consortium is able to degrade this bicyclic NA under methanogenic conditions.

3.5.3.6 Summary of the experiments involving NAs

The ability of NAs to support methanogenesis in the fine tailings samples was tested under a variety of conditions. The mixtures of NAs, both commercial and extracted from the MLSB, failed to stimulate methanogenesis, even after prolonged incubation. In some cases the mixtures of NAs were found to inhibit methanogenesis when present in high concentrations (150, 300 and 500 mg/L). In some situations, inhibition that occurred early in the experiment was overcome in time and methanogenesis continued, suggesting

that the microbial consortium is well able to adapt to these inhibitory compounds. Toxicity studies indicated that H_2-utilizing methanogens were directly affected by commercial and extracted NA mixtures. The toxicity data for the acetate-utilizing methanogens was not as conclusive, but it was determined that after the consortium recovers from the initial toxicity, acetate utilization by the methanogens is not hampered. Work with surrogate NAs indicate that degradation under methanogenic conditions is dependent on the compound, concentration and inoculum used. For example, at certain concentrations 3-cyclohexylpropanoic, 5-cyclohexylpentanoic and 6-phenylhexanoic acid were susceptible to degradation using sewage sludge. However, degradation of these compounds using fine tailings samples was only moderately successful. One of the three samples tested degraded 3-cyclohexylpropanoic acid and 4-cyclohexylbutanoic acid, and only at concentrations of 200 mg/L. The potential for NAs in the MLSB to be the main methanogenic substrate is unlikely. Individual components of the *in situ* mixture may be susceptible to degradation as suggested by the surrogate NA data, however, there appears to be more readily utilizable substrates available in the fine fine tailings which are being preferentially utilized.

3.6 Study of the potential relationship between SRB and methanogens within the fine tailings samples

SRB are known to be efficient competitors for acetate and/or and H_2. Methanogens generally have to wait until sulfate concentrations are depleted before gaining access to available substrate. Since the SRB and methanogen populations in the fine tailings samples were both large and since SRB can use a wilder range of substrates (Zinder 1993) than methanogens, it was thought that the SRB might provide acetate or CO_2 to the methanogens. Methanogens are unable to degrade lactate, while some SRB will readily utilize it (Hansen 1993). Complete mineralization of lactate by sulfate reduction produces carbon dioxide (equation [3.1]), however the degradation of lactate can follow other routes. If sulfate is present, SRB will degrade lactate to acetate (equation [3.2]), and when sulfate is absent other species will ferment lactate to propionate and acetate (Postgate 1984).

Propionate-forming bacteria prefer lactate as a carbon source and will degrade it via the acrylate pathway (equation [3.3]) or the succinate-propionate pathway (equation [3.4]) to produce propionate (Gottschalk 1986). Most propionate-producing microorganisms use the second pathway. A few bacteria, including *Clostridium propionicum*, use the acrylate pathway. Propionate is further degraded by SRB to acetate (equation [3.5]) but only when sulfate is available. The produced acetate can either be used by SRB known as complete oxidizers (acetate-utilizing SRB) or can be released into the surrounding environment by incomplete oxidizers which are unable to use acetate. The fine tailings samples were shown previously (section 3.2.4.1) to contain lactate-, acetate- and propionate-utilizing SRB in equal numbers (10^4 to 10^5/g) (Table 3.10).

$$CH_3CHOHCOO^- + 1.5SO_4^{2-} + 4H^+ \Rightarrow 3CO_2 + 1.5H_2S + 3H_2O \quad \text{equation [3.1]}$$

$$2CH_3CHOHCOO^- + SO_4^{2-} \Rightarrow 2CH_3COO^- + 2CO_2 + S^{2-} + 2H_2O \quad \text{equation [3.2]}$$

$$3CH_3CHOHCOO^- \Rightarrow 2CH_3CH_2OO^- + CH_3COO^- + CO_2 + H_2O \quad \text{equation [3.3]}$$

$$CH_3CHOHCOO^- + NADH + H^+ + ADP + P_i \Rightarrow$$
$$CH_3CH_2OO^- + NAD^+ + ATP + H_2O \quad \text{equation [3.4]}$$

$$CH_3CH_2OO^- + 3H_2O \Rightarrow CH_3COO^- + CO_2 + H_2O + H_2 \quad \text{equation [3.5]}$$

Theoretically, if the SRB are providing acetate to the methanogens within the fine tailings samples, then the addition of lactate would enhance this relationship and the addition of inhibitors would highlight the interactions between the two populations. Thirteen different treatments were established and monitored for the production of methane, the loss of lactate and the production and loss of acetate and propionate. Lactate was added to the appropriate microcosms as a carbon source to a final concentration of 890 mg/L. Some microcosms received sulfate to stimulate SRB activity, others received molybdate to hinder the activity of SRB and others received a methanogenic inhibitor, either CH_3F or lumazine. July 1998 samples MBC1-8 and MBC2-15 were both tested and produced identical results. For simplicity, only data from MBC1-8 has been presented.

Although previous work had shown that 4400 mg/L (20 mM) molybdate inhibited both sulfate-reducing and methanogenic activity in the fine tailings sample (section 3.4.3) molybdate was still chosen but used in a lower concentration. Tanka and Lee (1997) had shown than 660 mg/L (3 mM) molybdate would effectively inhibit SRB without any effect on methanogenesis, so the lower concentration was used. If the methanogens are dependent solely on the activity of the SRB for substrates, then the addition of the molybdate would retard the activities of the SRB, which would deplete methanogens of substrate and stop methanogenesis. If, however, other microorganisms are providing substrate to the methanogens, the addition of molybdate may depress but would not prevent methanogenesis.

Addition of lactate to the microcosms resulted in the production of acetate within the first week of incubation, and was coupled to the depletion of lactate in the microcosms (Table 3.25 and Figure 3.22). Acetate concentrations peaked between day 13 and day 20, after which concentrations began to drop. Methane production in the unsupplemented and lactate-supplemented control microcosms began around day 27, after acetate concentrations had peaked (Figure 3.22A and B). Interestingly, the unsupplemented controls (Figure 3.22A), which had no detectable acetate at time zero, demonstrated production and then loss of acetate which was accompanied by methanogenesis. This is the first time acetate concentrations have been measured in microcosms containing fine tailings. That methanogenesis began after acetate concentrations peaked and started to decline, in the unsupplemented controls, suggests that it is the breakdown of larger compounds to acetate that drives methanogenesis *in situ*. If acetate is produced *in situ* and is the main precursor of methane, it is surprising that the concentration of acetate in the microcosms at the first day of analysis was 0 mg/L. Perhaps, CO_2 reduction in the presence of H_2 is the primary methanogenic pathway and transferring the samples to microcosms triggers acetate metabolism. Perhaps H_2 concentrations are affected in the closed microcosm system or there is a disruption of interspecies H_2 transfer within the consortium due to agitation of the

sample. Monitoring of acetate concentrations in fresh fine tailings samples would help determine what is occurring *in situ*.

In the lactate + sulfate microcosms, the initial sulfate concentration was measured at 1280 mg/L and concentrations dropped rapidly to 120 mg/L by day 34. Sulfate concentrations remained around 120 mg/L for the remainder of the experiment. Addition of sulfate delayed methanogenesis until day 41 (Figure 3.22E), which was after sulfate concentrations were depleted (day 34). The SRB out-competed the methanogens for the acetate as they had a competitive advantage (sulfate present) and at this stage it is unlikely that the SRB are providing substrates to the methanogens. Perhaps *in situ*, when TEAs are lower, the SRB degrade other compounds leaving acetate for the methanogens.

Lactate was depleted the fastest in the sulfate amended microcosms (within 6 days) (Table 3.25 and Figure 3.22E), whereas addition of molybdate delayed lactate utilization until day 27 or 34 (Figure 3.22C and 3.22D). Sulfate stimulated the SRB causing rapid lactate consumption. Molybdate inhibited the SRB, decreasing the rate of consumption of lactate. The unsupplemented control (Figure 3.22A) and the lactate + sulfate supplemented microcosms (Figure 3.22E) produced identical amounts of methane. This may suggest that all the acetate generated from the added lactate was used by the SRB and when methanogenesis began after sulfate levels dropped, the methanogens scavenged any substrates remaining from the inoculum. Considering the stoichiometry of lactate mineralization via sulfate reduction (equation [3.1]) in the lactate + sulfate amended microcosms, this hypothesis is valid. These microcosms had a culture volume of 25 mL and initially contained 1280 mg/L sulfate and 630 mg/L lactate, which equals 0.33 and 0.18 mmole of sulfate and lactate (per microcosm), respectively. Based on equation [3.1] it would require 0.27 mmole sulfate to consume the added lactate leaving 0.06 mmole sulfate. The complete mineralization of lactate to CO_2 would consume most of the sulfate present, leaving any available substrates originally present in the inoculum for the methanogens. This would suggest that the SRB present are complete oxidizers. Alternatively, the SRB

Table 3.25: Summary of methane production, acetate production and consumption and lactate consumption in microcosms containing July 1998 sample MBC1-8 and sulfate, lactate and/or molybdate.

	control	890 mg/L lactate	lactate + molybdate	lactate + sulfate + molybdate	lactate + sulfate
CH_4 production started (day)	27	27	55	55	41
CH_4 production plateaued (day)	62	62	97	97	55
Final CH_4 (% vol)	9.6	17	1.7	0.90	10
Peak acetate production at (day)	13	13	20	13	20
Peak acetate concentration (mg/L)	1250	2600	1160	2890	4020
Acetate depleted by (day)	34	34	>125[a]	>125[a]	82
Lactate depleted by (day)	0	20	27	34	6

[a]acetate was not completely depleted at day 125 when the experiment was terminated, these microcosms contained ≈600 mg/L acetate

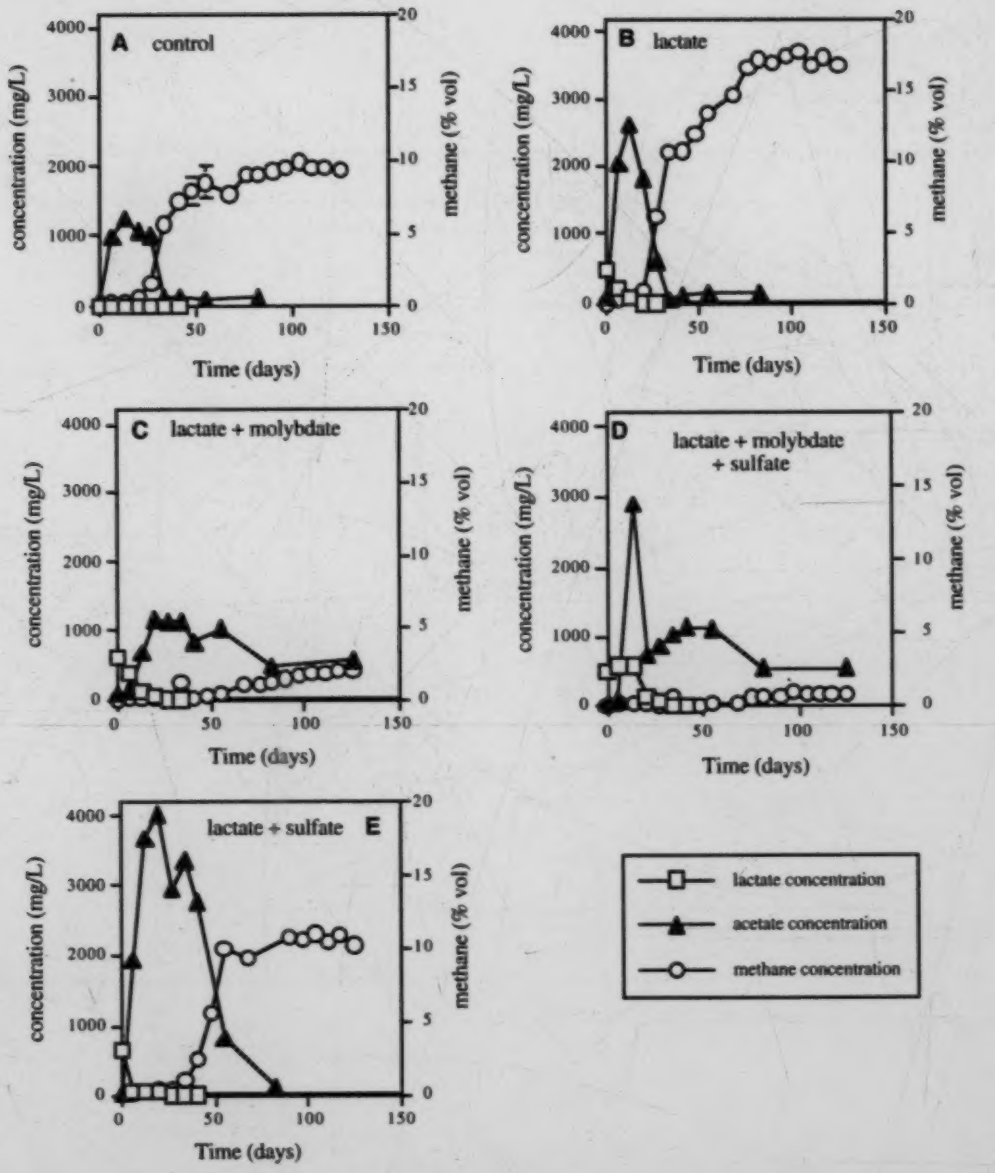


Figure 3.22: Lactate, acetate and methane concentrations in microcosms incubated at room temperature and containing July 1998 sample MBC1-8. Microcosms received no supplements (A), 890 mg/L lactate (B), lactate + molybdate (C), lactate + molybdate + sulfate (D) or lactate + sulfate (E). Each point represents the mean of triplicate microcosms.

would probably consume the substrates available in the inoculum and in so doing would deplete some sulfate. During degradation of the lactate, sulfate concentrations would drop, allowing methanogenesis to occur. The acetate available for the methanogens from the breakdown of lactate would likely be equivalent to the concentration of readily utilizable substrates originally present in the inoculum.

Sulfate was not depleted in the lactate + sulfate + molybdate microcosms but remained near the initial concentration of 1280 mg/L throughout the experiment (Table 3.25). In these microcosms, lactate was consumed to produce acetate and after peaking at 2890 mg/L, the acetate concentration dropped to near 600 mg/L by day 125, and methane did not exceed 1% vol (Figure 3.22D). This acetate production, consumption and plateau trend was the same for microcosms which received lactate + molybdate and no sulfate (Figure 3.22C). Consequently, the reduction of sulfate was not necessary for the consumption of lactate or acetate. Since the SRB were inhibited by molybdate, as indicated by the constant sulfate concentrations, other members of the consortium must have converted lactate to acetate, likely through fermentation. Acetate was present but not converted to methane (as noted by the low % vol CH_4 values), so molybdate also inhibited the methanogens. As both the SRB and methanogens were inhibited, the acetate in the lactate + sulfate + molybdate microcosms (Figure 3.22D) was consumed by other organisms in the consortium.

Propionate was also detected in the microcosms (Figure 3.23). A small amount of propionate was produced in the unsupplemented controls but much more was detected in the microcosms containing lactate. Although most SRB will degrade lactate to acetate, some will degrade it to propionate which in turn is converted to acetate and carbon dioxide (equations [3.3], [3.4], and [3.5]). Propionate accumulated in the microcosms containing molybdate likely because the SRB were inhibited and unable to convert the propionate to acetate. Propionate was produced and then depleted in the sulfate-supplemented microcosms, as sufficient sulfate was available to allow the propionate-utilizing SRB to

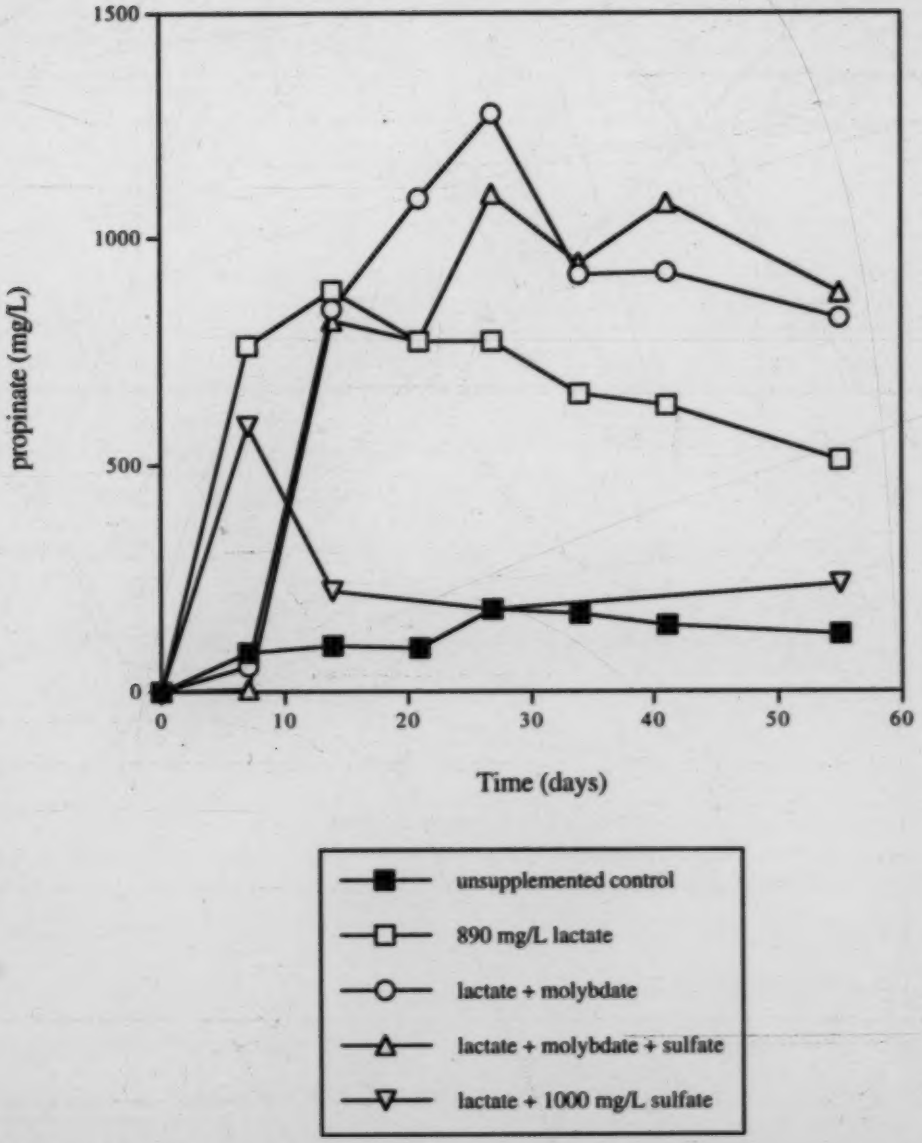


Figure 3.23: The production and loss of propionate in microcosms containing July 1998 sample MBC1-8 and lactate, molybdate and/or sulfate. Each point represents the mean of triplicate microcosms.

degrade the propionate to acetate. The partial loss of propionate in the microcosms containing only lactate likely occurred because not enough TEA was present to convert the propionate to acetate. Had sulfate been added to these microcosms, the propionate concentration would have dropped.

Qatibi et al. (1990) studied the degradation of lactate by a mixed bacterial culture from an anaerobic fermenter fed with distillery wastewater. They found that in the presence of sulfate and sulfate + molybdate, lactate was quickly consumed to produce acetate and propionate, which accumulated. If only sulfate was present, only acetate accumulated with propionate being consumed. These results are consistent with the data presented here: lactate consumption resulted in propionate and acetate production and when molybdate was present, propionate accumulated, although acetate was consumed in the microcosms studied here. Furthermore, propionate degradation was complete when sufficient sulfate was present. Unlike Qatibi et al. (1990), who found that sulfate did not affect methane production, the data presented here indicate that the presence of sulfate did inhibit methanogenesis. Qatibi et al. (1990) concluded that acetate played a minor role as an electron donor in sulfate reduction, being used solely by methanogens. The work here indicates that within the fine tailings consortium, acetate can be readily utilized as an electron donor for both SRB and methanogens, and when given sufficient sulfate the SRB will readily use acetate.

The use of methanogenic inhibitors was intended to determine whether acetate would accumulate, indicating that the SRB were providing methanogens with substrate. CH_3F, an analogue of methane, was chosen as it had been shown to prevent methanogenesis in pure cultures of aceticlastic methanogens and in mixed methanogenic cultures. Concentrations of 1 kPa were found to inhibit acetate-utilizing methanogens without affecting H_2- or formate-utilizing methanogens, SRB, acetogens or fermenters, thus providing a very specific inhibitor (Janssen and Frenzel 1997). The second methanogenic inhibitor, lumazine, was considered as it is a structural analogue of novel co-

factors involved in methanogenesis and has been found to disrupt the terminal steps of the methanogenic pathway at concentrations around 0.6 mM (Nagar-Anthal et al. 1996). At the concentrations used, neither of these compounds inhibited methanogenesis (Figure 3.24).

CH_3F had no effect on lactate degradation or acetate production. The slight delay in methanogenesis by 7 days in lactate-supplemented microcosms (Figure 3.24A) was due to a 7 d delay in acetate consumption. Perhaps CH_3F did slightly affect the acetate-consuming methanogens, but this inhibition was quickly overcome. CH_3F concentrations remained close to the original values for most of the experiment, with concentrations dropping slowly after day 60 to 50% of the initial concentration at day 90. CH_3F is a gas and as the microcosms were incubated stationary, it is possible that there was insufficient mixing of the gas into the sample, preventing the CH_3F from effectively inhibiting the methanogens.

Lumazine had no effect on the consortium in the microcosms (Figure 3.24B). The lumazine-amended microcosms had identical results to the unsupplemented control microcosms in all parameters tested. In both sets, methane production started on day 27 and plateaued on day 62 and acetate concentrations for both peaked at day 13 and were depleted by day 34 (data not shown). The work by Nagar-Anthal et al. (1996) using lumazine was performed on pure cultures and the extrapolation of inhibitory concentrations to environmental samples may be misapplied. The assumption that 0.6 mM lumazine would be able to affect the methanogens in the complex matrix of the fine tailings was wrong.

Although the compounds used failed to inhibit methanogenesis, corroborative data were obtained. The microcosms which received the inhibitor + sulfate were all delayed in their production of methane (Figure 3.24) until after sulfate concentrations were depleted at day 34. Furthermore, lactate consumption was accelerated in the presence of sulfate with levels dropping by day 6, while microcosms without sulfate had detectable lactate until day 20 to 40. Thus, the SRB population are quite capable of oxidizing the lactate supplied, and when sulfate concentrations are high, scavenge any available acetate present preventing the methanogens from active metabolism.

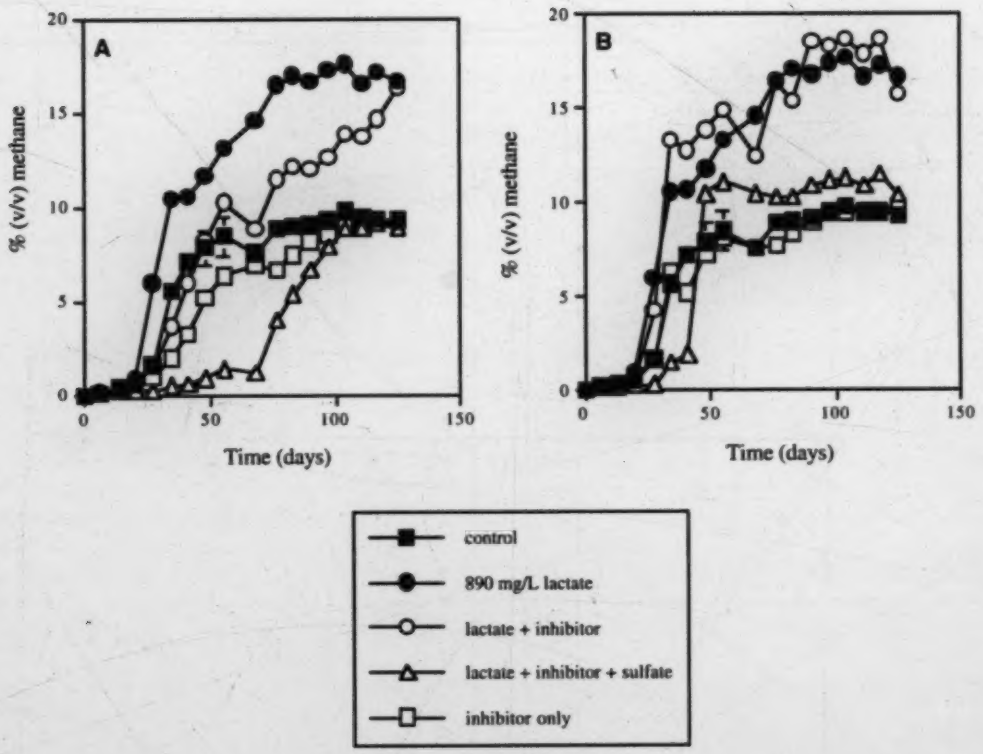


Figure 3.24: The effect of CH_3F (A) and lumazine (B) on methanogenesis in microcosms containing July 1998 sample MBC1-8 incubated at room temperature. Points represent mean values of triplicate microcosms and error bars for the controls represent one standard deviation.

While competition is generally held to be the most common relationship occurring between SRB and methanogens, there are at least two other types of possible interactions including co-existence and synergism (Smith 1993). Competition is historically the best understood and most studied of the types of interactions and is classified as an interaction between two groups for limiting electron donors. SRB out-compete methanogens for common electron donors because they have a greater affinity for both H_2 and acetate and gain more energy from the breakdown of these substrates than do methanogens (Capone and Kiene 1988). However the competitive success of SRB is severely dependent on the availability of sulfate (Lovley and Klug 1986). A variety of studies have demonstrated the ability of SRB to out-compete methanogens for available acetate and H_2 (Banat 1983; Capone and Kiene 1988; Lovley and Klug 1986; Yavitt and Lang 1990, Zhou and Fang 1998). Co-existence of SRB and methanogens is possible when they use different sources of electron donors. For example, Oremland and Policin (1982) documented that if sediment slurries received acetate and H_2, methanogenesis increased but was prevented if sulfate was also added. When methanol, trimethylamine or methionine were added, methanogenesis was again stimulated and the addition of sulfate had no effect on methanogenesis; these substrates had no effect on the activity of SRB. Therefore, it was concluded that the methanogens and SRB were co-existing by using different electron donors. Other studies of SRB and methanogens suggest that co-existence is a competitive relationship, turned non-competitive because the concentration of available substrates is high enough to support both populations, generally occurring in environments where there is high concentration of organic peat or sediment (Holmer and Kristensen 1994; Martens and Klump 1984; Yavitt and Lang 1990). Synergism or the interaction in which one group provides the electron donors for the other, is another possible relationship occurring between SRB and methanogens. The process of interspecies H_2 (or acetate) transfer is the best example of synergism. In environments where there are low concentrations of sulfate, SRB have been shown to ferment lactate to acetate and H_2, both of which are methanogenic substrates

(Bryant et al. 1977; Gunnarsson and Ronnow 1982; Mountfort et al. 1980). For lactate fermentation to proceed to H_2, a thermodynamically unfavorable reaction, H_2 must be removed from the system. In this case, H_2-utilizing methanogens aid SRB by depleting the produced H_2. Studies with sewage sludge (Ueki et al. 1992) and the anaerobic digestion of animal waste (Ueki et al. 1989) have shown a synergism involved in the degradation of propionate. In these studies, SRB degraded propionate to acetate which was metabolized only by the methanogens. Inhibition of methanogenesis resulted in the accumulation of acetate as the SRB failed to metabolize acetate. In this system, the SRB were directly providing the methanogens with substrate (acetate).

From the work presented here, it is clear that the SRB are able to utilize lactate, propionate and acetate, consistent with the enumeration of these populations, and that when sulfate is present, the SRB will actively out-compete the methanogens for acetate. However, sulfate concentrations *in situ* are low in the fine tailings samples (Table 3.1 and 3.2) and while the potential for competition exists, it is not likely the main interaction between the two populations. Unfortunately due to the failure of the methanogenic inhibitors to stop methanogenesis and the inhibition by molybdate, little information regarding the potential co-existence or synergism between these two populations was obtained. Both types of interactions are still valid possibilities. The tailings pond contains a high concentration of organic matter (both dissolved and particulate). It is possible that the pool of useable substrates is large enough to support both populations to minimize competition or to provide more than one source of electron donors. Synergism is an even greater possibility when the low concentration of sulfate in the fine tailings samples is considered. It may be that the SRB are fermenting organic molecules to produce acetate and H_2 (Postgate 1984) and are relying on the methanogens to remove the by-products through interspecies H_2 (acetate) transfer. Studies using radiolabelled lactate may provide more concrete evidence in determining the type of relationship occurring between the SRB and methanogens. However, it is unlikely that this question will be fully answered until the

substrates for methanogenesis are better understood. When specific compounds are identified, then further studies could reveal how the SRB and methanogens are interacting.

One of the questions originally posed at the outset of this project was, what conditions lead to the start of methanogenesis in the fine tailings? The ability of SRB to effectively out-compete the methanogens for available acetate in microcosms amended with sulfate suggests that low sulfate concentrations *in situ* have enabled methanogenesis to proceed. Sulfate concentrations in the MLSB throughout the early and mid 1980s ranged between 200 and 235 mg/L in the water zone (0 to 9 m), 60 and 120 mg/L in the shallow fine tailings (11 to 16 m) and 35 and 70 mg/L in the deep fine tailings (17 to 23 m) (MacKinnon 1989). During this time period, methanogenesis was not detected in the MLSB. In 1991, sulfate concentrations in the water were still in the mid-200 mg/L range but the shallow and deep fine tailings samples had dropped to between 30 and 40 mg/L and <20mg/L, respectively (MacKinnon and Sethi 1993). It was after 1991 that methanogenesis was first detected in the MLSB and since then has increased dramatically. Sulfate concentrations remain low in the fine tailings samples with <20 mg/L detected in 1997 and 1998 (Tables 3.1 and 3.2). The correlation between decreasing sulfate concentration and increased methanogenesis in the MLSB, plus the demonstration in microcosm studies that added sulfate eliminated methanogenesis, provide good evidence that methanogenesis in the fine tailings was delayed until sulfate concentrations decreased.

3.7 Estimating methane yields from fine tailings

Three sets of microcosms were monitored for over a year to determine the amount of methane yielded from a given amount of fine tailings sample. Large microcosms containing 250-mL fine tailings were prepared with August 1997 samples MB1-5, MB2-5 and MBIP1-5 that had been stored at 4°C for 6 mon. Two microcosms were prepared using each sample; one microcosm was incubated at room temperature, and the other at 14°C. After 3 mon, additional microcosms were prepared using the August 1997 samples containing 100-mL fine tailings. Four replicate microcosms containing sample MB1-5,

MB2-5, MBIP1-5 or DP-5 were incubated at both room temperature and 14°C. When the microcosms were prepared the samples had been stored at 4°C for 9 mon. Microcosms containing 100-mL fine tailings were also prepared with samples collected in July 1998. Five replicate microcosms were incubated at both room temperature and 14°C. These microcosms were prepared within 12 d of sample collection and contained samples MBC1-5, MBC1-8, MBC2-5, MBC2-15 or MBWIP-10.

The volume of methane produced increased over time (Figures 3.25 to 3.27). Methane accumulated faster in the microcosms incubated at room temperature (Figure 3.26). Methane volumes plateaued after day 300 for the August 1997 samples in the 250-mL microcosms incubated at room temperature and after 400 d for those incubated at 14°C (Figure 3.25). Methane levels plateaued after 300 d and 400 d in the 100-mL microcosms incubated at room temperature (Figure 3.26A) and 14°C (Figure 3.27A), respectively. The July 1998 samples plateaued in methane production after 320 d (MBWIP-10) and 450 d (other samples) when incubated at room temperature (Figure 3.26B). The microcosms incubated at 14°C did not produce methane, except for MBWIP-10 which plateaued after 320 d (Figure 3.27B).

A value for methane yield for each sample (Table 3.26) was determined by averaging a few of the individual values measured after methane production had plateaued. Since methane levels did not plateau at the same time for all of the microcosms nor were they all monitored for the same amount of time, each of the averaged values was calculated using a different number of observations. Data from the August 1997, 250-mL and 100-mL microcosms were averaged after day 400, with data from six time points used for the 250-mL microcosms and four for the 100-mL microcosms. Methane yield values for the July 1998 100-mL microcosms was averaged over the last two time points at day 446 and 486. Standard deviations (S) for these values were calculated using the formula: $S_{average} = (S_1^2 + S_2^2 + S_3^2 ...)^{1/2}$ (Skoog and West 1976).

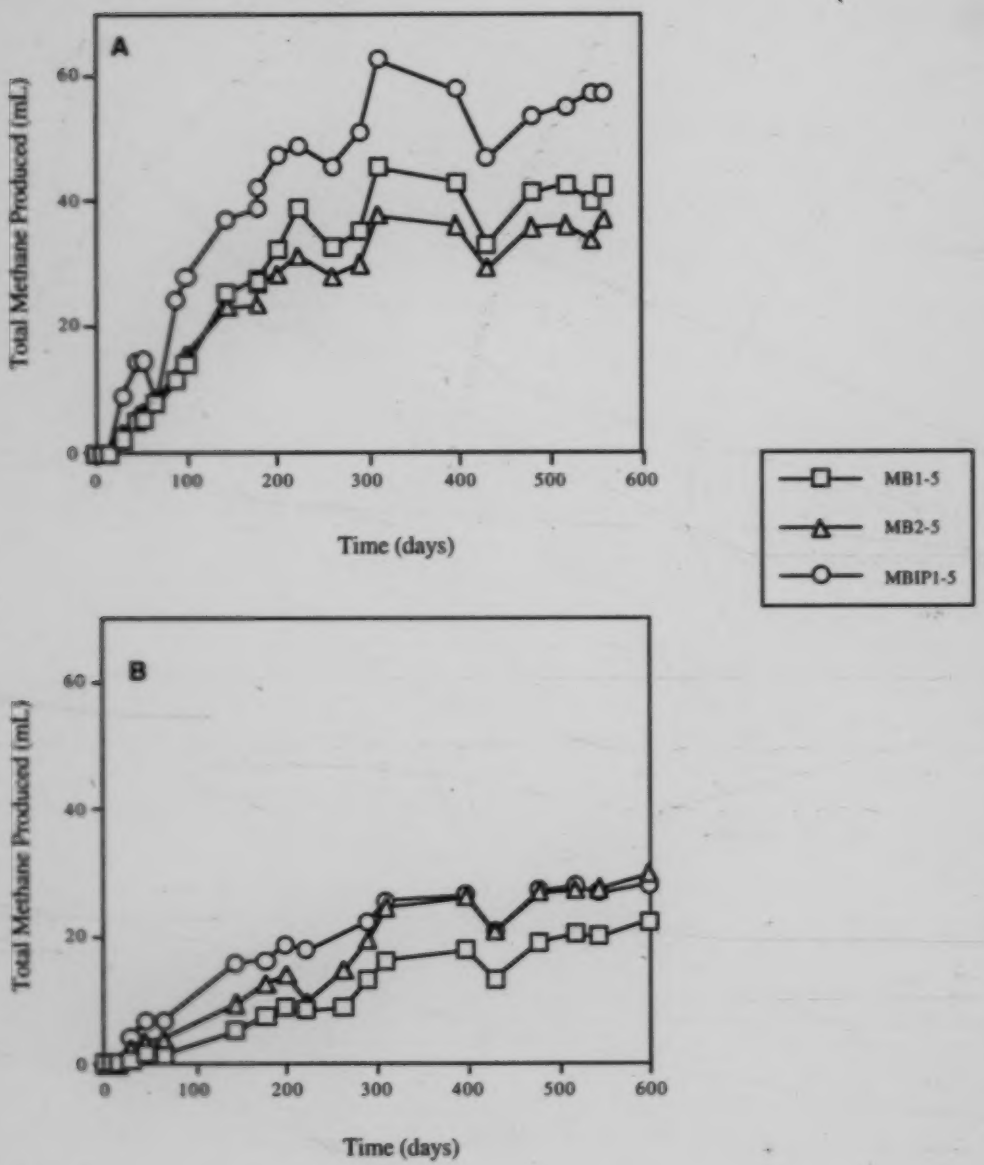


Figure 3.25: Methane produced in 250-mL microcosms inoculated with August 1997 fine tailings samples and incubated at room temperature (A) and 14°C (B). Each point represents one microcosm.

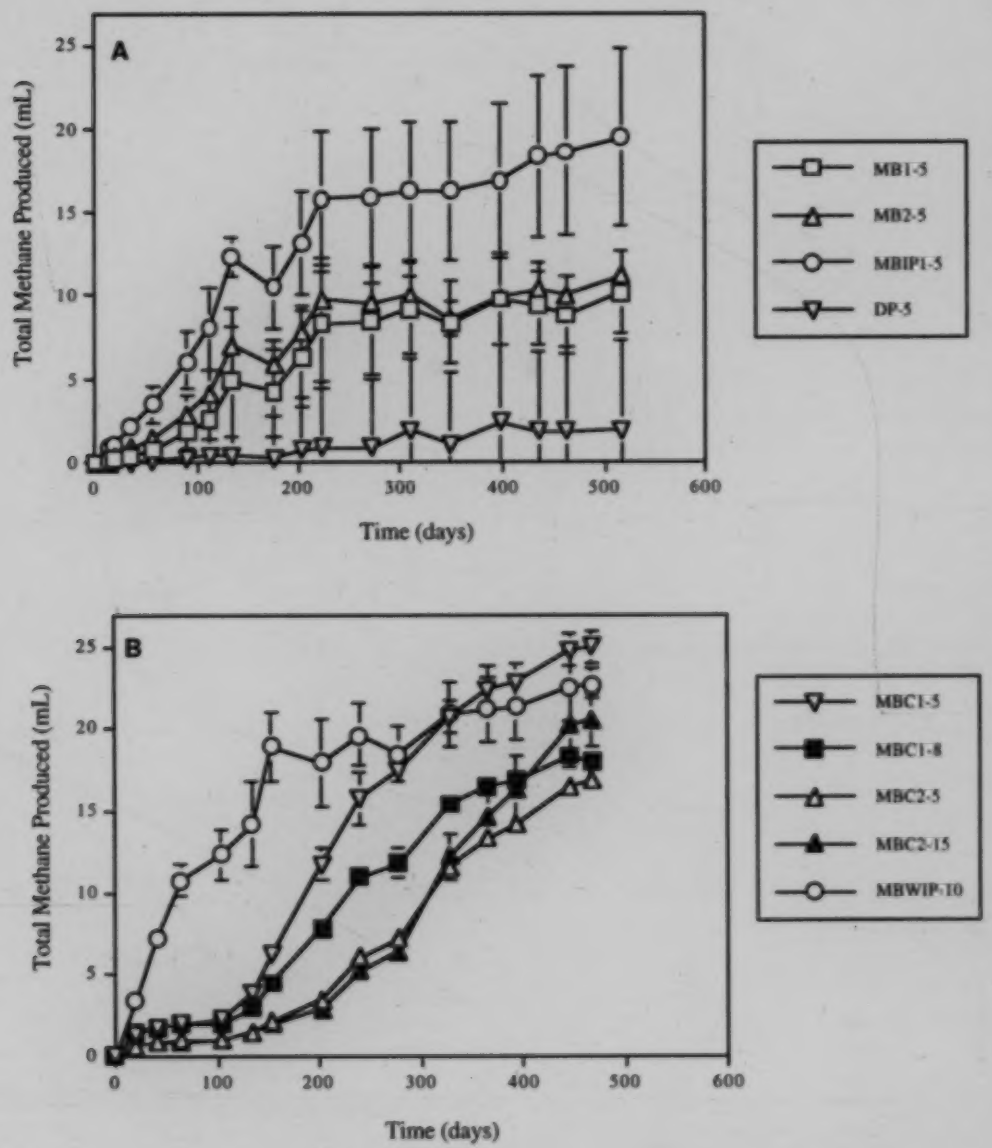


Figure 3.26: Methane produced in 100-mL microcosms inoculated with August 1997 (A) and July 1998 (B) samples and incubated at room temperature. Each point represents the mean of triplicate microcosms and the bars represent one standard deviation.

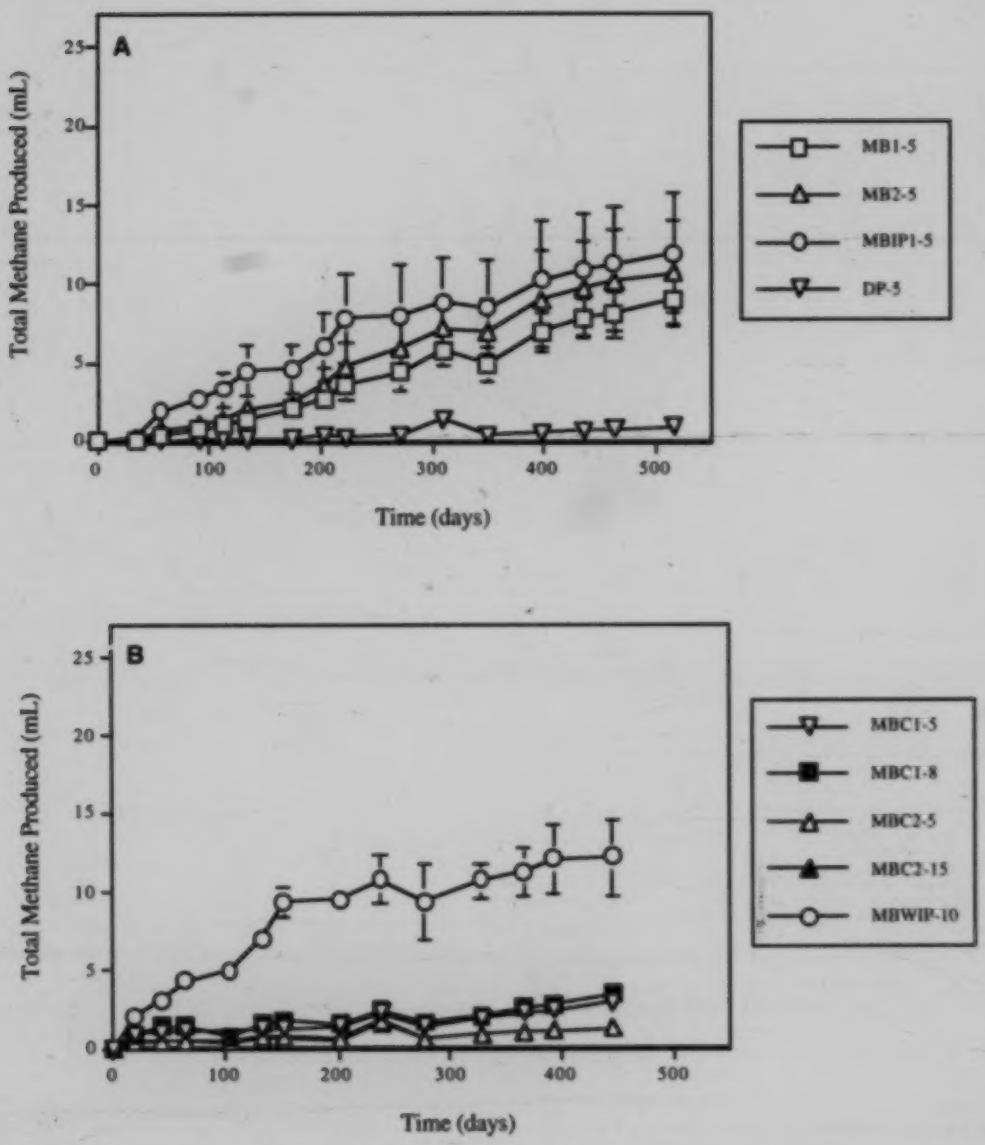


Figure 3.27: Methane produced in 100-mL microcosms inoculated with August 1997 (A) and July 1998 (B) samples and incubated at 14°C. Each point represents the mean of triplicate microcosms and the bars represent one standard deviation.

Methane yields were variable, ranging from 0.38 to 1.1 mL CH_4/g for the room temperature microcosms, and 0.04 to 0.51 mL CH_4/g for the microcosms incubated at 14°C (Table 3.26). The methane yields (mL CH_4/g) were compared using the Students' T Test (Harris 1991) at 95% a confidence interval. Statistical comparisons of methane yields were made between: incubation temperatures, samples from a given sample period, 250-mL and 100-mL microcosms containing August 1997 samples, and samples from different sample periods.

Statistical comparisons indicated that for each August 1997 sample, the 250-mL and 100-mL microcosms produced the same amount of methane on a per gram basis. The extra three months of storage of the August 1997 samples at 4°C, before they were used to inoculate the 100-mL microcosms, did not have an effect on total methane production. Furthermore, each of the samples MB1-5, MB2-5 and MBIP1-5 produced statistically similar amounts of methane at both incubation temperatures, and incubation at 14°C did not produce statistically different methane yields than room temperature incubation. Since the methane yield values did not differ significantly, the range of methane produced per gram of fine tailings sample is 0.33 to 1.00 mL CH_4/g (Table 3.26). While each sample produced a different volume of methane, the amount of methane produced on a per gram basis was not different among the August 1997 samples (Figures 3.25, 3.26A, and 3.26A).

Methanogens can tolerate temperature changes within certain limits, but show adaptation to an optimum temperature (Conrad and Wetter 1990) which is generally higher than *in situ* temperatures (Zeikus and Winfrey 1976). The production of methane in the room temperature incubated microcosms is consistent with the behavior of methanogens to adapt to increased temperatures. The rate of methanogenesis is generally lowered when incubation temperatures drop below 20°C because methanogens lose their competitive advantage against acetogens for H_2 (Conrad et al. 1989; Conrad and Wetter 1990; Schink 1997; Zeikus and Winfrey 1976). Furthermore, studies with ^{14}C-glucose have shown that

Table 3.26: Methane produced in microcosms containing August 1997 and July 1998 samples and monitored for over a year. Dry weight determination are reported in Appendix A. Sample identification includes the temperature that microcosms were incubated at, either at room temperature (RT) or 14°C. ± indicates one standard deviation from the mean value.

Sample and incubation temperature	Volume of sample used (mL)	Total incubation time (days)	Dry CH_4 produced at STP (mL)	Mass of fine tailings used (g, dry wgt)	CH_4 yield (mL/g)
MB1-5 RT[a]	250	556	41 ± 3.7	37	1.1 ± 0.10
MB2-5 RT[a]	250	556	35 ± 2.9	65	0.54 ± 0.04
MBIP1-5 RT[a]	250	556	55 ± 4.1	79	0.70 ± 0.05
MB1-5 14°C[a]	250	596	19 ± 3.1	37	0.51 ± 0.08
MB2-5 14°C[a]	250	596	26 ± 3.1	65	0.40 ± 0.05
MBIP1-5 14°C[a]	250	596	26 ± 2.7	79	0.33 ± 0.03
MB1-5 RT	100	516	9.6 ± 5.1	15	0.64 ± 0.34
MB2-5 RT	100	516	10 ± 1.6	26	0.38 ± 0.06
MBIP1-5 RT	100	516	18 ± 9.9	32	0.56 ± 0.31
DP-5 RT	100	516	2.0 ± 3.0	40	0.05 ± 0.07
MB1-5 14°C	100	516	7.9 ± 2.9	15	0.53 ± 0.19
MB2-5 14°C	100	516	9.9 ± 6.2	26	0.38 ± 0.24
MBIP1-5 14°C	100	516	11 ± 7.3	32	0.34 ± 0.23
DP-5 14°C	100	516	0.74 ± 0.59	40	0.02 ± 0.01
MBC1-5 RT	100	468	25 ± 1.4	26	0.96 ± 0.05
MBC1-8 RT	100	468	18 ± 0.56	24	0.75 ± 0.02
MBC2-5 RT	100	468	17 ± 1.0	26	0.65 ± 0.04
MBC2-15 RT	100	468	20 ± 3.1	35	0.57 ± 0.09
MBWIP-10 RT	100	468	23 ± 2.3	23	1.00 ± .010
MBC1-5 14°C	100	446	2.4 ± 0.34	26	0.10 ± 0.01
MBC1-8 14°C	100	446	2.8 ± 1.3	24	0.12 ± 0.05
MBC2-5 14°C	100	446	1.2 ± 0.23	26	0.044 ± .009
MBC2-15 14°C	100	446	1.1 ± 0.10	35	0.031 ± .003
MBWIP-10 14°C	100	446	12 ± 3.7	23	0.52 ± 0.16

[a]single microcosms established, all others prepared in triplicate

lowering the temperature decreases the rate of methanogenesis because the rate of metabolism by other members of the consortium, which provide methanogenic substrates, have slowed (Zeikus and Winfrey 1976). Methane production in the microcosms incubated at 14°C likely took longer to plateau because both the rate of methanogenesis and the rate at which methanogenic substrates were being provided had slowed.

Sample DP-5 was monitored in the 100-mL microcosms. It produced significantly lower amounts of methane than the other samples studied. DP-5 generated between 0.02 and 0.05 mL CH_4/g, and the temperature of incubation had little effect (Table 3.26). Fine tailings samples collected from the Demonstration Pond had the lowest methanogen numbers (10^4/g) and were the least active sample collected. Therefore, it is not surprising that the amount of methane produced in these microcosms was low compared to the samples taken from the active tailings ponds.

July 1998 samples were monitored in 100-mL microcosms. Incubation of these samples at room temperature (Figure 3.26B) produced higher methane yields than the incubation at 14°C (Figure 3.27B). Unlike the August 1997 samples, there were statistical differences among the methane yields for the different samples when incubated at room temperature (Table 3.26). Samples MB1-5 and MBWIP-10 yielded the same amount of methane (1.0 mL CH_4/g), more than MBC1-8 which produced 0.75 mL CH_4/g and MBC2-5 and MBC2-15 which both produced around 0.60 mL CH_4/g (Table 3.26). These values are within the 0.33 to 1.0 mL CH_4/g range observed for the August 1997 samples which were not considered statistically different. The standard deviations among the replicates for the July 1998 samples were smaller than the August 1997 samples and the range of statistical significance was narrower. Thus, the methane yields were statistically different in July 1998 and not in August 1997. However, further comparison between the August 1997 samples and the July 1998 samples incubated at room temperature indicated that the differences noted between the July 1998 samples were not significant when compared to the August 1997 samples. Consequently all the methane yields fall within a statistically

indistinguishable range of 0.55 and 0.96 mL CH_4/g with the exception of MB2-5 which only produced 0.38 mL CH_4/g. The lack of variability between the 1997 and 1998 data indicates that storage of the August 1997 samples at 4°C prior to the preparation of the microcosms had no significant effect on methane yield. Therefore, the pool of usable substrates was not significantly depleted with storage.

Incubation of the July 1998 samples at 14°C considerably hampered methanogenesis in the fine tailings samples, with the exception of MBWIP-10 (Figure 3.27B). Methane production in the MLSB samples did not increase over the 486 d incubation period and the methane yields were very low (0.03 to 0.10 CH_4/g) (Table 3.26). Methanogenesis was not depressed by incubation at 14°C in the August 1997 samples which had been stored before use (Figure 3.27A) but was hindered in the recently collected samples (Figure 3.27B). Why this occurred is unknown. The pool of usable substrates is not a limiting factor, as methanogenesis occurred in the room temperature incubated microcosms (Figure 3.26B) and 14°C is near the *in situ* mean temperature of the fine tailings and should allow methanogenesis to continue. The resazurin in these microcosms remained reduced, indicating that anaerobic conditions were not compromised. This temperature effect was only observed in the samples collected from the MLSB (MBC1-5, MBC1-8, MBC2-5 and MBC2-15) and not in the sample collected from the Base Mine Lake (MBWIP-10), which yielded statistically similar amounts of methane as its replicate microcosm incubated at room temperature.

Studies involving temperature effects on methanogenesis have shown that decreasing incubation temperature of methanogenic samples in laboratory microcosms (Conrad et al. 1989; Conrad and Wetter 1990; Zeikus and Winfrey 1976) and *in situ* (Beeman and Suflita 1990) lowers the rate but does not prevent methanogenesis. In systems where H_2 is the dominant electron acceptor, dropping the temperature allows acetogens to outcompete the methanogens for H_2 (Conrad et al. 1989; Schink 1997; Zeikus and Winfrey 1976) because the H_2 threshold for the acetogens decreases more substantially

than for methanogens at lower temperatures (Conrad and Wetter 1990). The acetogens convert H_2 and CO_2 to acetate causing a shift to acetate-consuming methanogenesis. Successful competition of the acetogens for H_2 is contingent on low nitrate and sulfate levels (Conrad et al. 1989) and low acetate concentrations, which makes the acetogens dependent on the activities of aceticlastic methanogens (Schink 1997).

Little work has been reported on the extent of methanogenesis at low temperatures. The rate of methanogenesis may be lowered but is the extent of methanogenesis affected by temperature? Studies of peatlands by Updegraff et al. (1998) demonstrated that methanogenesis slowed when temperatures were lowered but that the total amount of methane produced was not affected by temperature. Work by Kotsyurbenko et al. (1996) suggest that when a methanogenic community is balanced with H_2 and CO_2 as a substrate, methane is the endpoint of metabolism, with acetate being produced as an intermediate when temperatures drop to 10°C. Incubation temperatures less than 20°C may shift H_2 consumption from methanogenesis to acetogenesis, but the acetate produced is a methanogenic substrate and the pool of useable substrates for methanogenesis should not be reduced. Consequently, total methane produced in the 14°C-incubated microcosms after prolonged incubation should be the same as those incubated at room temperature which was observed in the 250-mL (Figure 3.25) and 100-mL (Figure 3.26A and 3.27A) microcosms containing the August 1997 samples.

On the final day of analysis for the 250-mL microcosms incubated at room temperature (day 556) the microcosms were shaken on a reciprocating shaker for 5 min at 188 reciprocations/min. After shaking, the microcosms were allowed to settle for 5 min and a gas measurement was taken. This procedure was repeated twice and after each disturbance neither the volume of gas nor the CH_4 (% vol) in the microcosm headspace changed, suggesting that there was little or no trapped methane in the samples. Subsamples of the slurry (50 mL) were collected from each of the 250-mL room temperature microcosms and sent for dissolved gas composition analysis (Maxxam Analytics Inc.,

Edmonton, AB). *In situ*, methane is trapped in the layers of fine tailings in the MLSB, thus if methane was trapped within the sample in the microcosms, it would lead to inaccurate methane yield values. The gas analysis revealed that there was between 0.81 and 1.7 mL methane in the slurry portion of the 250-mL microcosms. These values are less than 2% of the total methane produced by these microcosms and do not constitute a significant reserve of unaccounted for methane. Consequently, the methane yields reported are not biased by any large amounts of methane trapped in the fine tailings within the microcosms.

Using the anaerobic biodegradability assay of Young (1997), Sobolewski (1999) determined that an inoculum consisting of a mixture of five fine tailings samples would produce 0.075 mL CH_4/mL fine tailings after 158 d incubation. Although the temperature of incubation was not indicated in that report, the assay is generally performed at 35 to 37°C. The data here were presented in mL CH_4/g fine tailings dry weight. However, conversion of the methane yields gave a range of 0.10 to 0.25 mL CH_4/mL fine tailings (Table 3.27) for the samples collected in August 1997 and July 1998 when incubated at room temperature. In the study by Sobolewski, the microcosms were incubated for 158 d whereas after 150 d incubation of the microcosms monitored in this study, methane concentrations were still increasing (Figures 3.25 to 3.27), so it is not surprising that the value reported by Sobolewski (1999) is lower than the yields presented here. It is suggested that the methane yield values presented here more realistically demonstrate what would happen *in situ*. The microcosms were monitored for a longer period of time, used more fine tailings sample (250 and 100 mL versus 1 mL) and were incubated near the *in situ* temperature.

From the 0.10 to 0.25 mL CH_4/mL fine tailings methane yield range obtained, the potential amount of methane to be produced by the MLSB at its current volume of around 300 x 10^6 m^3 fine tailings is predicted to be 30 to 75 x 10^6 m^3. To produce 0.10 to 0.25 mL CH_4/mL fine tailings, the amount of carbon used is 54 to 134 µg/mL fine tailings. For example, 0.10 mL CH_4/mL fine tailings x mmol CH_4/22.4 mL CH_4 x 12 mg C/mmol CH_4

x 1000 μg/mg = 54 μg C/mL fine tailings. The DOC values for the fine tailing samples (Table 3.1 and Table 3.2) averaged to 50 mg C/L fine tailings. The amount of DOC per mL of fine tailings is equivalent to 50 μg C. The DOC concentration (50 μg C/mL fine tailings) is less than the carbon needed to generate the amount of methane produced in the microcosms (54 to 134 μg C/mL fine tailings). It appears that some amount of undissolved organic carbon, needs to be solubilized before it is converted to methane.

There was no analysis performed for total organic carbon however, a majority of the carbon in the fine tailings is from the bitumen present. Assuming that bitumen is 75% carbon, and is the primary, but not only source of carbon in the tailings ponds, we can approximated how much of the total carbon in the tailings pond is being converted to methane. If the fine tailings contain on average 2% (w/v) bitumen, then there are 20 g bitumen/1000 mL fine tailings. Multiplying this by 75% and converting to mg carbon, there are approximately 15 mg C/mL fine tailings. Comparing to the amount of carbon (54 to 134 μg C/mL fine tailings) needed to produce the measured volume of methane in the microcosms, released methane accounts for less than 1% of the total carbon in the fine tailings.

The estimated methane yields (Table 3.26) are lower than the total amount of methane to be produced per gram of fine tailings because of the residence time of the samples in the tailings ponds. It is not known how long the samples were in the tailings ponds prior to collection. The residence time would affect how much methane was already produced by the collected material. The methane yields generated in the laboratory may only represent a fraction of the methane actually produced by a given volume of fresh fine tailings.

At the time of sampling, subsamples were collected and analyzed for trapped gases (Table 3.27). Low O_2 concentrations confirm that there was little O_2 contamination during handling. The amount of CH_4 trapped in the MLSB fine tailings samples varied between 1 and 53 mL CH_4/L fine tailings (0.001 to 0.53 mL CH_4/mL fine tailings). The Base Mine

Table 3.27: Compositional analysis of the gases trapped in fine tailings samples used to determine methane yield per mL fine tailings. Fine tailings samples were collected from the MLSB, Base Mine Lake and Demonstration Pond in August 1997 and July 1998. Data were provided by M. MacKinnon, Syncrude Canada Ltd. and analyses were performed by Maxxam Analytical. Values are expressed as mL gas/L fine tailings.

Year	Sample	Depth (m)	CH_4 mL/L	N_2 mL/L	CO_2 mL/L	O_2 mL/L
1997	MBC1-5	5	8	21	4	1
	MBC2-5	5	1	16	2	0.7
	MBIP1-5	5	17	30	13	0.1
	DP-5	5	0.2	5	2	0.2
1998	MBC1-5	5	10	27	13	1
	MBC1-8	8	53	57	31	1
	MBC2-5	5	14	31	14	0.2
	MBC2015	15	12	73	7	0.1
	MBWIP-10	10	73	47	45	2

Lake Samples (MBIP1 and MBWIP) had 17 and 73 mL CH_4/L fine tailings trapped and the Demonstration Pond had the least amount of trapped methane with 0.2 mL CH_4/L fine tailings. The variations in the values of trapped gas result from different rates of methane production, hence the lowest value for the Demonstration Pond samples, but may also result from different rates of methane evolution. Factors mediating the release of trapped gas from the fine tailings deserves further study. Does a sample have to become saturated with gas before it releases the methane it has trapped or are there other processes involved? Why do some samples have more trapped gas than others and how long will methane continue to evolve from the fine tailings?

Methanogenic substrates will continue to be provided as the volume of the MLSB increases. However, once inputs into the system stop, substrates will be depleted and the rate of methane production will slow. Many factors will likely affect how long methanogenesis will proceed *in situ*, but it will not continue indefinitely, likely less than 10 to 20 years after inputs into the MLSB stop. This is assuming: that the rate and extent of methanogenesis occurs uniformly throughout the fine tailings and are similar to what was observed in the microcosms; that after methanogenesis plateaus, there are no more organics available that could serve as sources of methanogenic substrates, that is adaptation within the consortium will not allow formerly recalcitrant organic compounds to be degraded to H_2 or acetate. Although methanogenesis will likely stop when substrates are depleted, a significant portion of the methane produced will likely be trapped in the fine tailings and continue to evolved over time. How long it will take before a majority of the methane has evolved from the tailings ponds is unknown.

4. Future Work

There are many possible avenues in which continued work with the fine tailings samples can be pursued. One aspect would be to identify whether CO_2 reduction or acetate consumption is the major source of methane *in situ*. Both H_2- and acetate-utilizing methanogens were enumerated in the fine tailings samples but it would be interesting to know if one pathway dominates. Carbon and hydrogen stable isotope composition analysis of the methane produced by the samples would indicate which process was dominant. Reduction of CO_2 or metabolism of acetate would each produce a signature ^{13}C ratio which would indicate by which pathway the methane is produced. Understanding which precursor is the main methanogenic substrate will reveal whether the methanogens are mainly involved in interspecies H_2 or acetate transfer and may aid in determining what larger compounds are being degraded to provide methanogenic substrate. Work presented here showed that acetate and propionate are produced and consumed in unsupplemented control microcosms, monitoring acetate and other volatile acids *in situ* with fresh fine tailings samples would also be useful. It would also be interesting to determine if under different conditions, one pathway is preferred over the other which may give indications into whether shifts in the metabolism of the population occur.

Understanding whether H_2 or acetate is the main methanogenic precursor will help to determine what type of relationship is occurring between the SRB and methanogens. Understanding the relationship between SRB and methanogens is important for understanding how the two populations will respond under new conditions, for example at the oil sands operations where sulfate is added during the new CT process being used to reduce the volume of fine tailings.

The relationship between the SRB and methanogens can be further studied using better inhibitors. Molybdate was shown to inhibit both SRB and methanogens, and was not useful as a specific SRB inhibitor. Testing molybdate at a concentration lower than 3 mM may be effective in inhibiting SRB without affecting the methanogens. Other SRB

inhibitors, such as selenium and tungsten, have the same mechanism of inhibition as molybdate and therefore would unlikely provide more specific inhibition of SRB. Finding a suitable inhibitor for a complex environmental sample such as the fine tailings is difficult, as most work reported has been done with pure cultures. The two methanogenic inhibitors tested in this study did not work. Studies with fluoromethane may be repeated with the modification of shaking the cultures to facilitate dissolution of the gas into the sample which may enhance inhibition. As well, lumazine may be a more potent methanogenic inhibitor if tested at a higher concentration. The relationship study could be further modified by using ^{14}C-lactate. If $^{14}CH_4$ is produced then there would be a concrete link between the degradation activities of the SRB to produce methanogenic substrates, as methanogens are unable to degrade lactate and would therefore depend on SRB to metabolize the larger compound to acetate.

Focus of this study was primarily on the competitive relationship between methanogens and SRB due to the availability of inorganic TEAs, mainly sulfate. SRB are however able to use organic molecules as TEA through fermentation. Fermentative microorganisms might the primary supplier of methanogenic substrates and may be the key to understanding carbon and electron flow through the fine tailings consortium to methane.

It is also possible that the methanogenic consortium is degrading biomass created by the death of other microorganisms, such as SRB. To test this hypothesis, cultures of SRB could be supplemented with ^{14}C-lactate to produce ^{14}C-labeled cells. Then after a suitable incubation, the radioactive SRB could be harvested and added to microcosms with fine tailings. The microcosms would be monitored for the production of $^{14}CH_4$. If $^{14}CH_4$ was produced then it would have originated from ^{14}C in the SRB cells from their initial assimilation of ^{14}C-lactate. These studies could then be extended to include other substrates used by SRB and other fermentative bacteria.

The substrate studies initiated in this project focused only on the anaerobic degradation of particular compounds to produce methane. The complexity of the fine

tailings samples provides an endless list of possible methanogenic substrates which could be tested. One aspect that was not included in the substrate studies, which may occur *in situ*, is the possibility of primary attack of compounds by aerobic microorganisms. It is possible that aerobes are required to begin the metabolism of a recalcitrant compound. The metabolites from aerobic attack may be more susceptible to anaerobic degradation leading to methane production. For example, work presented here showed that methanogenic microcosms mineralized hexadecanoic acid to methane but these microcosms could not mineralize hexadecane. Hexadecanoic acid is an intermediate in the aerobic attack of hexadecane. The aromatic compounds and NAs, which were shown not to contribute to methanogenesis under strictly anaerobic conditions may yield methane if first attacked by the indigenous aerobic populations. To study this, microcosms could be prepared which contained an aerobic headspace and a layer of MLSB water covering the fine tailings inoculum. Once close, the aerobes would consume the oxygen pushing the system anaerobic and the production of methane could be monitored. The actual importance of aerobic degradation in the tailings ponds is not clear. Metabolites would have to transfer down into the fine tailings in order to contribute to methanogenesis. The feasibility of mass transfer downwards may be low since the tailings pond system has an upward energy flow as sedimentation and gas production occurs (M. MacKinnon, personal communication).

Continued enumeration of the microbial populations is another important area of investigation. It will be important to establish whether methanogenic populations in the MLSB have stabilized and whether they will continue to rise in the Demonstration Pond and the Base Mine Lake. Furthermore, it will be important to monitor how and if the sizes of SRB populations will change and how these changes relate to methanogen numbers and sulfate concentrations. It will also be a good idea to pursue a functional methanotroph enumeration assay with particular emphasis on how methanotroph numbers may be changing in the Demonstration Pond and how this affects dissolved oxygen values in the wetland.

The surrogate NA work was promising, showing that some surrogate NAs were susceptible to degradation under methanogenic conditions. Volumes of methane produced suggested that ring cleavage was occurring, therefore it would be important to pursue metabolite studies. This is the first report of 3-cyclohexylpropanoic acid, 4-cyclohexylbutanoic acid, and 5-cyclohexylpentanoic acid being degraded under anaerobic conditions. Detecting intermediates and understanding the pathway of degradation, would be important to establish. If ring cleavage of the surrogate NAs occurs it may suggest that cleavage of other saturated cyclic compounds or aromatic compounds is possible *in situ*.

Having shown that individual naphthenic-like compounds can be degraded by the fine tailings samples to methane may suggest that individual components of the *in situ* NA mixture are being degraded which may not have been evident in the microcosm studies. Using GC-EIMS (St. John et al. 1998) or ESIMS (Morales-Izquierdo 1999) it may be possible to monitor the change of Z family composition in microcosms. This work could be initiated with fine tailings samples depleted of useable substrate (those that have been left unsupplemented until methane levels plateau). The interference of high background methane levels could then be minimized and small changes in methane concentrations could be detected. Minimizing the headspace volume would also be modification.

Another aspect requiring further work is trying to estimate accurately, methane yields from the fine tailings samples. One of the problems faced in this project was not knowing how long samples had been in the MLSB prior to collection. More realistic methane yield values could be obtained by using fresh fine tailings collected as they enter the tailings pond (residence time is zero). Microcosms containing methanogenic fine tailings (residence time unknown) would serve as the control and would be compared to microcosms containing fresh fine tailings (residence time is zero) inoculated with methanogenic fine tailings (residence time unknown). The difference in methane production between the two sets of microcosms would give a better indication of the ultimate amount of methane to be produced per mL of fine tailings.

5. Concluding Remarks

Throughout this study, each of the main objectives of the project was addressed providing insight into the nature of methanogenesis in the fine tailings samples. There were seven objectives of this work, designed to address the questions: what started methanogenesis, what are the potential methanogenic substrates and how long will methanogenesis occur?

Objective one. To demonstrate methanogenic activity of Syncrude Canada Ltd. fine tailings samples in laboratory microcosms. Methanogenesis occurred in ALL unsupplemented microcosms. Addition of known methanogenic substrates, H_2 and acetate, stimulated methane production in all cases. Having established that methanogenesis could be sustained in laboratory microcosms, addition of various compounds facilitated studies to address the other objectives of this project.

Objective two. To enumerate methanogens and SRB in the Syncrude Canada Ltd. fine tailings samples. The 5-tube MPN assay was shown to be reliable for enumerating these microbial populations in the fine tailings samples. It was determined that both acetate- and H_2-utilizing methanogens were present in the samples, in equal abundance, ranging between 10^5 to 10^6/g therefore both substrates were likely available to the methanogens *in situ*. The Demonstration Pond was found to have the lowest numbers of methanogens (10^4 to 10^5/g), but these values appear to be increasing, and the Base Mine Lake samples had the largest population sizes (10^7/g). Continued enumeration will confirm whether population sizes have stabilized.

SRB were enumerated using lactate as an electron donor, and population sizes ranged from 10^5 to10^6/g, except in the samples collected from 5 m where counts were 10^7/g. The 5-m samples were the only ones in which SRB outnumbered the methanogens and it is at this depth that sulfate concentrations decrease from the high concentrations in the overlying water. The reduction of sulfate provides SRB with a competitive advantage over the methanogens. In addition to lactate, the SRB were shown to grow in the presence of

other electron donors including H_2, benzoate, acetate and propionate. MPN values using the alternate electron donors were essentially the same; populations ranged between 10^4 to 10^5/g, suggesting that each of these different substrates are available to the SRB *in situ*. Since the methanogens and the SRB are inhabiting the same environment and have large population sizes there must be sufficient substrate available for both.

Objective three. To enumerate methanotrophs in water samples to determine if methane production is increasing the numbers of these methane-oxidizing bacteria. Difficulties with the enumeration of these hard-to-culture bacteria failed to provide information regarding this population. This work was abandoned early in the project.

Objective four. To determine if the onset of methanogenesis was caused by a decrease in SRB activity. Studies using molybdate at 3 and 20 mM failed to specifically inhibit the SRB in the samples. Molybdate at these two concentrations inhibited the SRB but also prevented methanogenesis, making it difficult to determine whether reducing the activity of the SRB in the samples would cause methanogenesis to increase. Studies with molybdate, sulfate and lactate suggested that when sufficient TEA was available, the SRB will actively degrade lactate to acetate and propionate and then continue to consume these intermediates. When sulfate was not present, methanogenesis proceeded. However, addition of sulfate delayed methanogenesis until sulfate was depleted. Inhibition of methanogenesis by sulfate addition was also observed in other studies pertaining to objective six. *In situ*, sulfate concentrations in most of the fine tailings samples are low (<20mg/L) and subsequently methanogenesis proceeds unimpeded. The ability of SRB to out-compete the methanogens for substrate when sufficient TEA is added suggests that methanogenesis was likely delayed in the MLSB until sulfate concentrations dropped, which allowed methanogenesis to proceed. That SRB are still present in the fine tailings samples suggests that a new relationship between the SRB and methanogens has been established.

Objective five. To determine in substrate studies which compounds will yield methane in Syncrude Canada Ltd. fine tailings samples. A variety of potential methanogenic substrates were studied including petroleum, aromatic compounds and NAs. The petroleum tested, Prudhoe Bay crude oil and oil sand bitumen, failed to stimulate methanogenesis under the experimental conditions used. Furthermore, a mixture of PACs including: naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, dibenzothiophene, phenanthrene, anthracene, fluorene, fluoranthene, biphenyl, 4-methylbiphenyl and benzothiophene did not stimulate methanogenesis. Experiments using ^{14}C-labelled toluene, naphthalene and phenanthrene did not produce $^{14}CO_2$ or $^{14}CH_4$, whose production would indicate mineralization of these aromatic compounds. Furthermore, ^{14}C-hexadecane was not utilized by the samples to produce $^{14}CH_4$ suggesting that long chain alkanes are not the main sources of methanogenic substrates within the fine tailings samples. If the microcosms used in the substrate studies failed to accurately reflect *in situ* conditions or ignored other potential influences such as initial aerobic attack, then there remains the possibility that these compounds could stimulate methanogenesis.

The fine tailings samples were able to degrade phenol, *m*-cresol and *p*-cresol but not *o*-cresol when incubated at room temperature. However the lag times before the phenols were mineralized to methane were long (70 to 150 days), longer than expected for compounds known to be susceptible to degradation under methanogenic conditions. Mineralization of the phenols did not occur at 14°C, a more environmentally suitable incubation temperature. It was suggested that at 14°C, the pool of usable substrates readily available in the fine tailings samples was not depleted during the experiment and the consortium did not have to switch to phenol degradation to obtain substrate. Consequently, phenols are not likely the main methanogenic substrates within the fine tailings sample, but if present, would be degraded to methane after more readily available substrates have been depleted.

Studies with NAs, including commercial and extracted NAs mixtures and an individual ^{14}C-labelled bicyclic NA, decahydro-2-naphthoic acid, failed to stimulate methanogenesis in the fine tailings. In many cases, commercial and extracted NA mixtures inhibited methanogenesis, especially when added in concentrations much higher than the 80 to 120 mg/L NAs *in situ* concentrations. With prolonged incubation the affected populations adapted to the toxic compounds, allowing methane production to proceed after initial inhibition. Toxicity studies indicated that the NAs mixtures (>90 mg/L) were directly inhibitory to H_2-utilizing methanogens. This toxicity was overcome with time as the methanogens adapted and methanogenesis proceeded. It was not conclusively determined whether the NA mixtures were directly toxic to the acetate-utilizing methanogens. However, after the consortium recovered, substrate utilization by the aceticlastic methanogens was not affected.

Studies with surrogate NAs indicated that successful degradation depended on the compound, the concentration of the compound, and the inoculum used. Sewage sludge was found to degrade 200, 400, 600 and 800 mg/L 3-cyclohexylpropanoic acid, 200 mg/L 5-cyclohexylpentanoic acid and 200 and 400 mg/L 6-phenylhexanoic acid, but not 4-cyclohexylbutanoic acid. Three fine tailings samples were tested for their ability to degrade the surrogate NAs, and only one sample, collected from the Base Mine Lake, had limited ability to degrade the surrogate NAs. MBWIP-10 degraded 200 mg/L 3-cyclhexylpropanoic acid and 200 mg/L 4-cyclohexylbutanoic acid, but degradation began only after the pool of usable substrates in the inoculum had been completely utilized. While the potential exists for individual naphthenate-like compounds to be degraded in the fine tailings samples, they are not likely providing a main source of methanogenic substrates. The amount of methane produced from the degradation of the surrogates was more than expected if only the carboxylic side chains were undergoing beta-oxidation. It appears that ring cleavage is occurring, but this will need to be confirmed with further work. This is the

first report of the degradation of 3-cyclohexylpropanoic acid, 4-cyclohexylbutanoic acid and 5-cyclohexylpentanoic acid under any anaerobic condition.

Objective six. To determine if methane production can be inhibited by the addition of TEAs, including sulfate and nitrate. The stimulation of competition for substrates by other microbial populations through the addition of TEAs inhibited methanogenesis. Nitrate ≥1800 mg/L and sulfate ≥ 5000 mg/L were the most effective concentrations to sustain inhibition in the fine tailings samples. Nitrate was the superior inhibitor. In the presence of nitrate, methanogenesis was inhibited immediately and required a lower concentration than required for sulfate-induced inhibition of methanogenesis. However, the use of nitrate to inhibit methanogenesis is not attractive because of the logistical problems and financial and energy costs associated with adding of this TEA plus the generation of N_2O, a greenhouse gas, from nitrate reduction. Therefore this approach does not appear to provide a good option for controlling methanogenesis in the fine tailings storage ponds. The inhibition of methanogenesis, in the presence of sulfate suggests that methanogenesis begins only after sulfate, is depleted and the activities of the SRB are decreased (see objective four). The addition of sulfate to the fine tailings in the CT process has yet to demonstrate sustained inhibition of methanogenesis.

Objective seven. To use the data collected to address the three original questions. What started methane production? It is likely that methanogenesis was suppressed until the concnetrations of TEAs of other more energy-efficient microbial populations dropped, allowing the methanogens to successfully compete for available substrates. More specifically, methanogenesis was probably delayed until sulfate was depleted but despite the low concentration of TEA, the SRB did not disappear from the MLSB. Apparently, a new or altered relationship may have been established between the methanogens and the SRB.

What are the potential substrates for methanogenesis? Although many different substrate experiments were initiated, none of them indicated what compounds within the

tailings pond are the main methanogenic substrates. The stimulation caused by the phenols and the surrogate NAs occurred only after the pool of readily available substrates already in the sample was depleted. Obviously, other more easily degraded compounds are the source of methanogenic substrates.

How long will methanogenesis continue? An estimate for this question was to be determined once the methanogenic substrates were known. Although the substrates were not identified, this question can be addressed by the long-term microcosms which were monitored for over a year for total methane production. In these microcosms, methane production slowed down within 200 to 400 d showing that in a closed system methanogenesis is a finite process. Fine tailings samples produced between 0.10 and 0.25 mL CH_4/mL fine tailings. This range is an underestimate of the actual amount of methane to be produced per mL of fine tailings because it was not corrected for how long the material was in the tailings pond prior to sampling. The total amount of methane to be produced will ultimately depend on the final volume of fine tailings accumulated. Once all inputs into the MLSB are stopped methanogenesis will continue until substrates are depleted. Although methanogenesis is in the fine tailings was shown to be a finite process there are many factors influencing how long methanogenesis will continue, but it could stop, or at least slow significantly, within 10 to 20 years after inputs into the MLSB stop. Although methane production will slow and eventually cease, much of the produced gas is likely to be trapped in the fine tailings. Methane evolution from the tailings ponds will be a much longer process.

Additional conclusions. Other observations were made that did not fall into one of the seven main objectives. An experiment was designed which tested the effect of prolonged storage at 4°C, 14°C and room temperature on the population sizes of H_2- and acetate-utilizing methanogens and SRB, and on the utilization of acetate by methanogens. Storage for up to 9 mon did not diminish the numbers of H_2- and acetate-utilizing methanogens or SRB, nor did it dampen acetate utilization by the aceticlastic methanogens.

Other microbial populations were not studied. If other members of the consortium which provide methanogenic substrates are affected by storage, then methanogenesis would be indirectly affected by storage. Additionally, the long term microcosms indicated that storage of the sample for up to 9 mon at 4°C does not greatly deplete the amount of substrate available *in situ*, as the values for total methane produced were not significantly different between the microcosms inoculated with stored or recently collected sample. This information will be valuable for future workers who might use microcosms to study microbial processes in fine tailings.

In some experiments, replicate microcosms were incubated at room temperature and at 14°C (an environmentally appropriate temperature). The same trends were noted between microcosms incubated at the different temperatures, but the lag before methanogenesis was initiated was delayed and the rate of methanogenesis was slower in the microcosms incubated at 14°C. For instance, utilization of acetate in supplemented microcosms generally stimulated methanogenesis after 20 to 25 d when incubated at room temperature and after 50 to 60 d when incubated at 14°C. The long term microcosms studied for total methane production suggest that the total amount of methane to be produced by microcosms, incubated at 14°C, is similar to the amount produced when incubated at room temperature. While the onset of methanogenesis in microcosms incubated at 14°C is delayed, the extent of methanogenesis is not altered.

Part of the motivation behind this project was understanding the possible effect the methanogenesis in the fine tailings samples was going to have on eventual remediation plans for the tailings waste, mainly the wet landscape approach. In this approach, the fine tailings, after being transferred to the old mined-out pit (presently the Base Mine Lake) would be capped with a layer of water to create a viable and self-sustaining wetland ecosystem. The Demonstration Pond is a small pilot project of this wet landscape approach. The creation and maintenance of a viable ecosystem over the last seven years provides credence to the potential usefulness of this remediation approach. However, methanogen

numbers appear to be increasing in this pond and the impact of this on the lake ecosystem from methane-oxidizing bacteria will need to be followed. Furthermore, the samples from the Base Mine Lake which will ultimately form the large-scale lake ecosystem, have higher methanogen counts than its source of fine tailings, the MLSB. That methanogen numbers are larger in the Base Mine Lake, suggests that the methanogen populations are increasing in this system and having a larger initial population of methanogens prior to water capping may affect the viability of a wetland ecosystem. Since the Demonstration Pond was established with fine tailings samples in 1993, when methanogen numbers were still relatively low and visible gas evolution (bubbles) had not begun on the MLSB, it is not actually an accurate model of the large-scale lake ecosystem planned for the Base Mine Lake. The success of the Demonstration Pond may not translate into success on a larger scale, since the fine tailings in the Base Mine Lake have a much higher concentration of methanogens. Active methanogenesis will continue to be a major consideration with respect to the wet landscape ecosystem remediation approach. Fortunately though, it has been shown that methanogenesis in the fine tailings samples is a finite process and will stop when substrates are depleted. Therefore, if the fine tailings in the Base Mine Lake are left untouched, methanogenic substrates will eventually be consumed. When methanogenic activity begins to drop, the water cap in the Base Mine Lake will be under less stress from the methane production in the fine tailings layer. However, continued methanogenesis in the Base Mine Lake, may lead to vast amounts of methane being released into the atmosphere from the fine tailings. Its impact on greenhouse gas emissions from oil sands operations will still need to be addressed.

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Appendix A: Dry weight determinations

The dry weight of the samples was determined for each of the fine tailings samples collected (Table A1). Ten milliliters of sample were pipetted onto a pre-weighed aluminum weigh boat, the weight was measured and the boat was put into a drying oven set at 125°C and the samples were dried to a constant weight. The dry weight (g)/mL was determined by subtracting the water loss from the sample weight and the value was then divided by the volume of sample used (10 mL). For example:

sample MBC1-10

wet sample:	10.63 g	wet sample:	10.63 g
dried sample:	- 4.36 g	empty boat:	- 1.00 g
water loss:	6.27 g	total sample:	9.63 g

$$\text{dry weight} = \frac{\text{weight of total sample - weight of water loss}}{\text{volume of sample}}$$

$$= \frac{9.63\text{ g} - 6.27\text{ g}}{10\text{ mL}} = 0.34\text{ g/mL sample}$$

Table A1: Dry weight determinations of the fine tailings samples collected.

August 1997[a]	g/mL	July 1998	g/mL
MB1-5	0.15	MBC1-5	0.26
		MBC1-8	0.24
MB1-10	0.37	MBC1-10	0.33
MB1-15	0.38		
MB1-20	0.43		
MB2-5	0.26	MBC2-5	0.26
MB2-10	0.34	MBC2-10	0.25
MB2-15	0.35	MBC2-15	0.35
MB2-20	0.33	MBC2-20	0.52
MBIP1-5	0.31	MBWIP-10	0.23
DP-5	0.40	98DP-5	0.37

[a]July 1997 samples M5, M10, and M15 had values of 0.27, 0.40 and 0.35 g/mL, respectively.

Appendix B: Estimating methane yields from fine tailings

B.1 Gas measuring apparatus

In order to determine the volume of methane produced in microcosms containing fine tailings samples, a gas volume measuring apparatus was set up as per Fedorak and Hrudey (1983) (Figure B1). The apparatus consisted of two Hamilton gas tight glass syringes (D and E), an 18-G x 1.5-inch needle (A), a manometer (F and G) and two 3-way Luerlok stopcocks (B and C) (Kontes Glass Company, NJ). The stopcocks, originally, 2-way, were modified by cutting away the small plastic external stop to enable a 360° rotation. The manometer consisted of a 10-mL volumetric pipette inserted through a two-hole stopper into a 125-mL Erlenmeyer flask partially filled with water and was connected to the syringes with Tygon tubing. After assembly, the apparatus was checked for gas leaks. To do so, the apparatus was removed from the stand and 8 mL of air was drawn into syringe D and stopcock B was positioned so that all three arms were open and stopcock C was positioned so that arm E and the arm to the manometer were closed. The needle was inserted deep into a rubber stopper and the apparatus was submerged under water. The plunger in syringe D was depressed to 2-mL mark, creating a pressure of 4 atm. Any leaks present would be manifested by the appearance and evolution of bubbles at the point source. The procedure was then repeated by drawing 8 mL air into syringe E. No leaks were found and routine checks were performed to ensure that the apparatus remained gas-tight.

B.2 Use of the gas measuring apparatus

Prior to use, the apparatus was flushed with O_2-free CO_2 in N_2 by drawing up gas into each of the syringes, followed by evacuation. This was repeated twice to ensure that no O_2 would enter the anaerobic microcosms from which measurements were being taken. After flushing, the needle was inserted into the microcosm with stopcock B closed to the

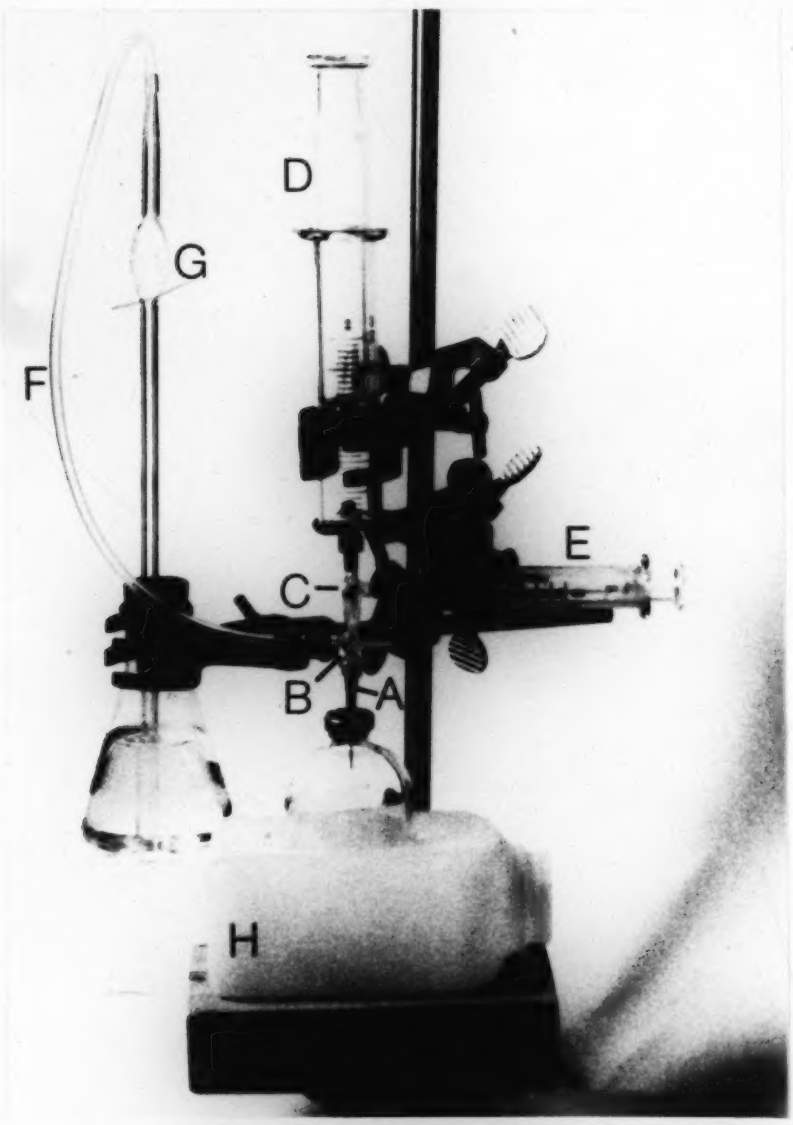

Figure B1: Picture of the apparatus used to measure total methane produced in microcosms (from Fedorak 1984).

manometer and stopcock C closed to syringe E. Excess pressure generated in the microcosm caused syringe D to move up, and the plunger was pulled up until pressure approached atmospheric and then an additional 5 mL of gas was removed from the microcosm (to prevent loss of gas when manometer was opened). If syringe D was not large enough to accommodate for the amount of excess gas produced then stopcock C was adjusted to open syringe E. While watching the water level in the manometer rise, stopcock B was adjusted slowly to open all three arms. If the water in the manometer was going to flow into the Tygon tubing, then the manometer was immediately closed off as too much extra volume in the syringes had been created. In these situations, the volume in the syringe(s) was slightly reduced. Afterwards, stopcock B was adjusted to open all three arms and the volume in the syringe(s) was adjusted until the manometer indicated the system was at atmospheric pressure as determined by the level of the meniscus in the pipette. Stopcock C was then opened and minor volume adjustments were made to ensure atmospheric pressure. Once atmospheric pressure had been established, the sum of the volume in syringes D and E indicated the volume of excess headspace gas produced in the microcosms.

A sample of headspace gas was then removed for GC analysis. The needle of a syringe was inserted into the microcosm and the amount of headspace required for analysis was withdrawn into the syringe. The system was adjusted to atmospheric pressure and the needle was removed and the headspace sample injected into the GC.

The volume of excess headspace gas in syringes D and E can be injected back into the microcosm or can be wasted to relieve pressure build up in the microcosm. If wastage is wanted, the desired amount is transferred to syringe E, after stopcock B has been rotated to close off the arm to the manometer. Stopcock C is used to close off syringe E and any remaining gas in syringe D is injected back into the microcosm. The microcosm is removed and stopcock C is opened to syringe E and the gas is discharged and the known volume is recorded.

During each measurement the following parameters were recorded: total volume of gas in syringes D and E, volume of gas removed for GC, volume of gas wasted, temperature and barometeric pressure.

B.3 Preliminary measurements with gas measuring apparatus

In order to become familiar with the apparatus and to test the reliability and repeatability of the measurements, preliminary tests were performed including air tests, and "dry" and "wet" methane tests. For each test, 158-mL serum bottles were sealed with rubber stoppers, crimped and punctured with two 18 G 1.5-inch needles and allowed to sit for at least 1 h to allow bottles to reach ambient temperature and pressure, after which the needles were removed. For the air test, a co-worker added various volumes of air to the equilibrated bottles which were then measured with the apparatus.

For the methane test, 15 mL of methane were added to the equilibrated bottles and measurements and samples for GC analysis were taken to determine methane concentration. Then 10 mL water were added to each bottle and shaken vigorously and let sit for at least 1 h. Gas measurements were then repeated on the "wet" bottles and samples were taken for GC analysis. The amount of dry methane volume was determined and compared to the expected values to determine % recovery. By adding 15 mL of methane to the "dry" serum bottles one would expect to measure 8.67% vol methane ([15/(15+158)] x 100), which corresponds to 13.8 mL dry CH_4 after accounting for STP and the appropriate correction factors. Since three 0.1-mL samples of headspace were taken for GC analysis from the "dry" bottles, the amount of methane expected to be remaining in the bottle for the "wet" bottles was 13.7 mL (13.75 mL - (0.3 mL x 8.67% vol CH_4 x 1/0.97)).

These tests were repeated a number of times and with repeated use of the apparatus, the reproducibility of the measurements increased and the deviations from the expected volumes dropped so that the data obtained were considered reliable. The results from the tests are presented in Figure B2 and Table B1.

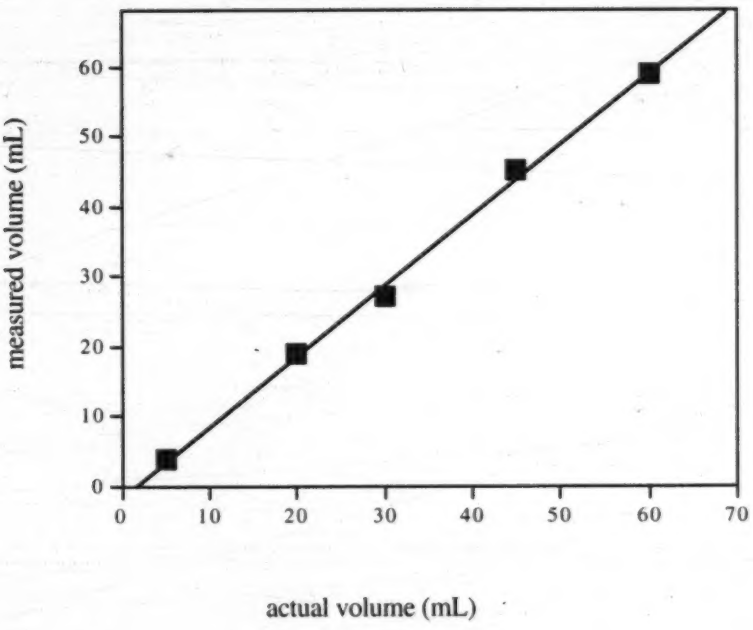

Figure B2: Measured volume of air in equilibrated 158-mL serum bottles that received different amounts of added air. Solid line represents expected values. The squares represent the experimentally determined values. Points represent single measurements.

Table B1: Measured volumes of displaced air, % vol methane, calculated volume of dry methane and % recovery in "dry" and "wet" methane tests.

Bottle	measured volume in "dry" bottles (mL)	% vol CH_4	volume of dry methane (mL)	% recovery	measured volume in "wet" bottles (mL)	% vol CH_4	volume of dry methane (mL)	% recovery
1	14.1	8.72	13.3	97	24.0	8.56	13.0	96
2	15.6	9.37	14.3	105	25.6	9.30	14.2	105
3	15.1	8.81	13.4	99	25.0	8.51	13.0	96
4	15.4	8.97	13.7	101	24.3	8.81	13.4	99
5	15.5	9.23	14.1	104	25.0	8.79	13.4	99
6	15.8	9.04	13.9	102	25.2	8.79	13.4	99
7	15.0	8.64	13.2	97	24.0	8.31	12.6	94
8	15.6	8.49	13.0	96	24.0	8.03	12.18	90
			ave:	100			ave:	97

Appendix C: Calibration curves for phenolic compounds

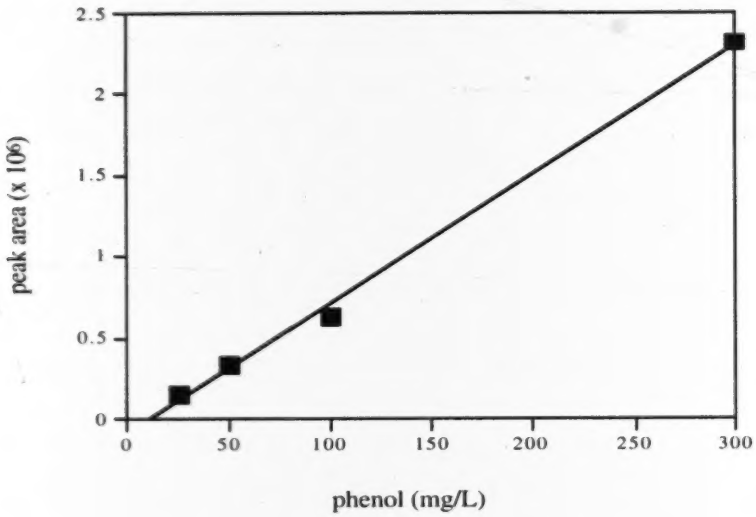

Figure C1: Calibration curve for phenol analysis by GC. Each point represents the mean of triplicate injections. Equation of the line is $y = 0.008x - 0.087$, $R^2 = .997$.

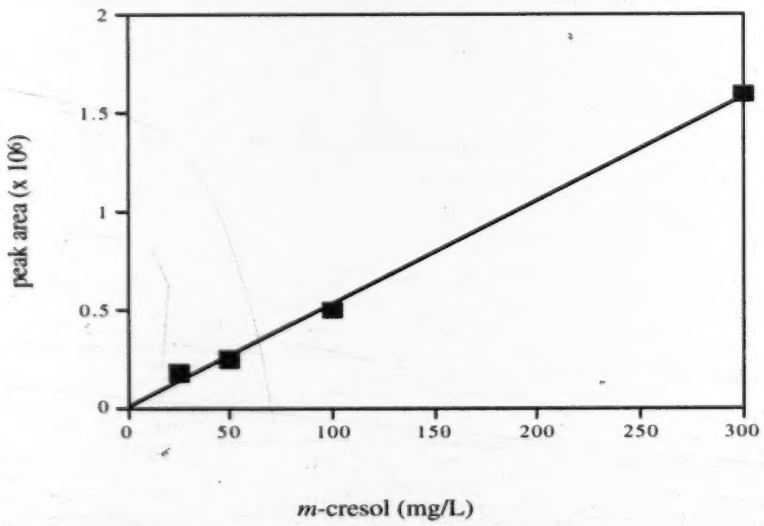

Figure C2: Calibration curve for *m*-cresol analysis by GC. Each point represents the mean of triplicate injections. Equation of the line is $y = 0.005x - 0.06$, $R^2 = 0.997$.

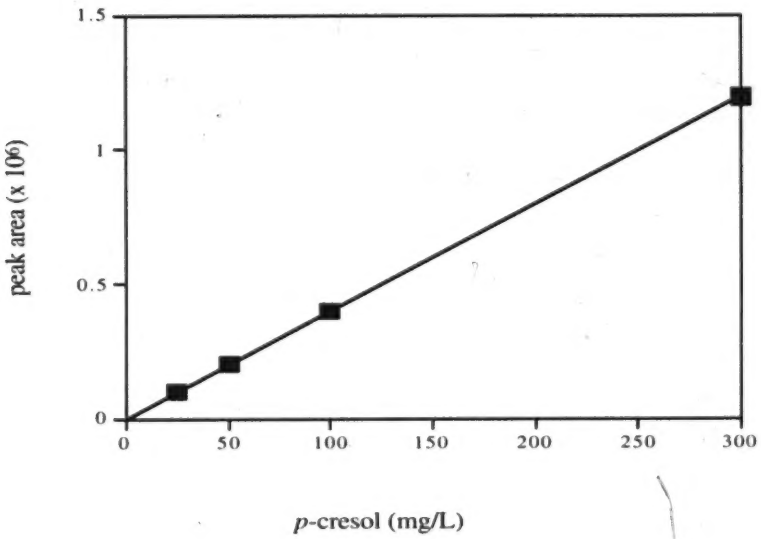

Figure C3: Calibration curve for *p*-cresol analysis by GC. Each point represents the mean of triplicate injections. Equation of the line is $y = 0.0043x + 0.011$, $R^2 = 1.00$.

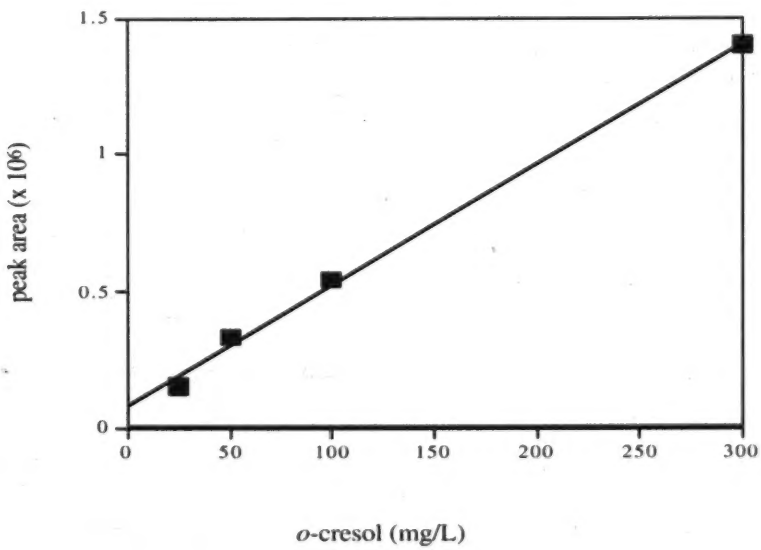

Figure C4: Calibration curve for *o*-cresol analysis by GC. Each point represents the mean of triplicate injections. Equation of the line is $y = 0.004x + 0.08$, $R^2 = .997$.

Appendix D: Calibration curve for sulfate analysis

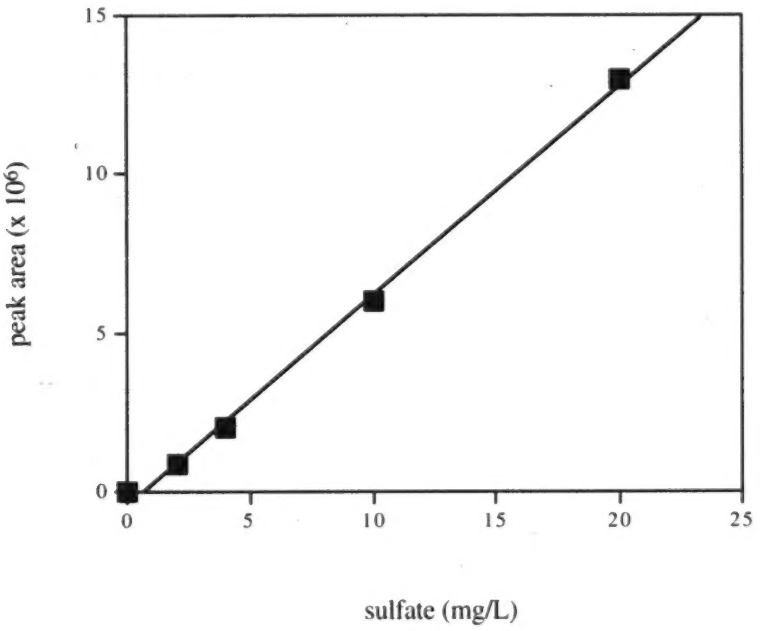

Figure D1: Calibration curve for sulfate analysis by ion chromatography as performed at the Limnology Laboratory of the Department of Biological Sciences at the University of Alberta. Each point represents the average of duplicate injections. The equation of the line is $y = 0.661x - 0.382$, $R^2 = .997$

Appendix E: Calibration curves for acetate and propionate analyses

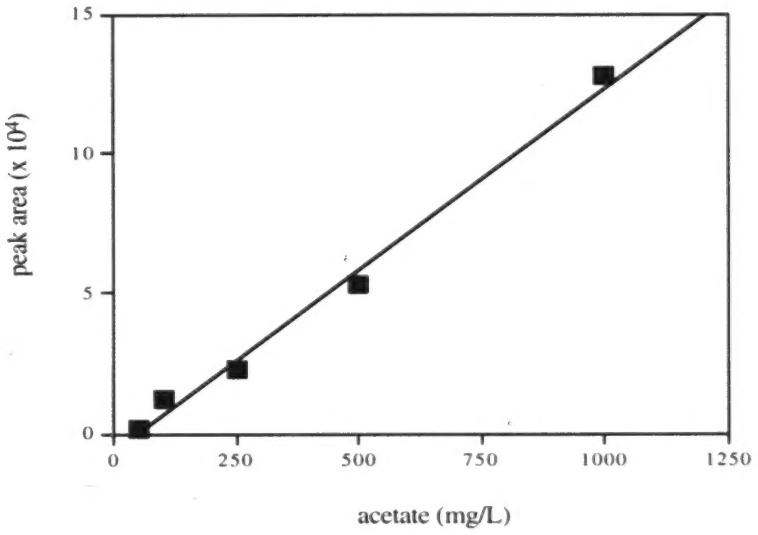

Figure E1: Calibration curve for acetate analysis by GC. . Each point represents the mean of triplicate injections . The equation of the line is $y = 0.013x - 0.66$, $R^2 = .995$

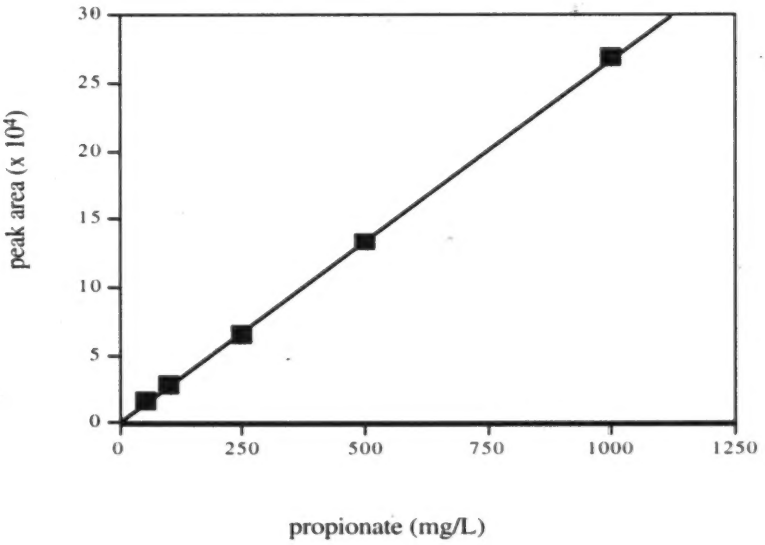

Figure E2: Calibration curve for propionate analysis by GC. Each point represents the mean of triplicate injections. The equation of the line is $y = 0.027x + 0.08$, $R^2 = 1.00$.

Appendix F: Calibration curve for lactate analysis

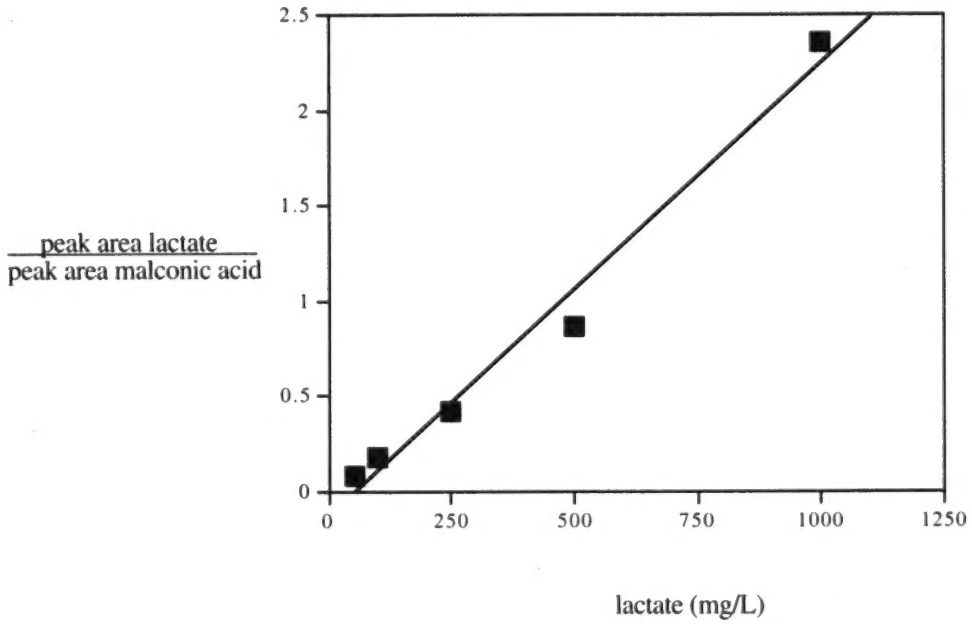

Figure F1: Calibration curve for lactate analysis by GC. Each point represents the mean of triplicate injections. The equation of the line is $y = 0.002x - 0.121$, $R^2 = .981$.

Appendix G: $^{14}CO_2$ and $^{14}CH_4$ trapping system development

Initial attempts to use a Packard gas proportional counter (GPC) model 894, which differentiated between and quantified headspace $^{14}CO_2$ and $^{14}CH_4$, were unsuccessful. Detailed examination of the GPC indicated that portions of the GPC internal system were corroded and could not be repaired. It was decided to use the internal connections and the functional oxidation oven of the GPC and modified trapping systems of Nuck and Federle (1996) and Fedorak et al. (1982) to create a new detection system.

In the first variation, two 2-vial traps were connected to each other with continuous tubing with the oxidation oven between them. The first set of vials would trap $^{14}CO_2$ and $^{14}CH_4$ would pass to the oxidization oven and be converted to $^{14}CO_2$ and be trapped in the second set of vials. The radioactivity trapped in ACS fluor in the vials could then be counted using a scintillation counter. In addition to the two scintillation vials used at each trap, an empty Hungate tube was added in series after the second vial to act as a safeguard against pressure build ups. Headspace samples were injected into a Varian model 700 GC. The CO_2 and CH_4 were separated by the column and a HP model 3390A integrator quantified and indicated when the CO_2 and CH_4 were eluting. The effluent from the GC was connected to the trapping system created in the body of the GPC and allowed to pass into the 2-vial traps.

After construction and gas leak testing, the flow rate of helium was set at 80 mL/min. However, flow rates through the trapping system were never constant and decreased with passage through the system. Despite extensive testing, the reason for the decreasing flow rates was not determined. Preliminary trials evaluated a variety of parameters to determine the optimal operating conditions for the detection system.

G.1 Detection of $^{14}CO_2$ and trapping efficiency

The ability of the 2-vial systems to trap $^{14}CO_2$ was tested using $H^{14}CO_3$. Fifty milliliters of water were added to three 125-mL serum bottles after which aliquots of a

$H^{14}CO_3$ solution prepared in a borax buffer (pH = 9) were added for a final radioactivity of 1.5 x 10^5 dpm. The bottles were sealed and 2 mL 4N H_2SO_4 was added to acidify the microcosm, releasing the label as $^{14}CO_2$; the amount of $^{14}CO_2$/mL headspace would be around 2000 dpm. Using pH paper, the microcosms were determined to have a pH between 0 and 1. One milliliter headspace samples were injected into the GC and after flushing, radioactivity was localized in trap 1A with counts ranging between 140 and 165 dpm. Although values are considerably lower than the expected count of 2000 dpm, one cannot assume that all of the $H^{14}CO_3$ was converted to $^{14}CO_2$. In order to determine how much $^{14}CO_2$ was in the headspace samples, in subsequent testing 1 mL samples were also injected directly into fluor and counted. Each set of traps was tested in isolation and found to be 100% efficient in trapping $^{14}CO_2$ (when compared to radioactivity counts obtained from directly injecting samples into fluor). However, when the efficiency of the second trap was tested in series with trap 1, that is a sample was injected and allowed to pass through empty vials in trap 1 and through the oxidation oven into trap 2, little radioactivity was recovered in the second trap. This indicated that radioactivity was being lost somewhere between traps 1 and 2.

The first consideration was that the flow rate of helium (70 mL/min) through the system was too fast for efficient trapping of the label in the second set. Replicate samples were measured at flow rates of 70 mL/min, 50 mL/min and 30 mL/min. Decreasing the flow rate had no effect on the amount of radioactivity recovered in the second set of vials.

The next consideration was that the duration of flushing affected the recovery of radioactivity in the second trap when connected in series with the first. Flushing times were increased from 3 to 5 min which resulted in greater recovery of radioactivity in the second traps. It was hypothesized that the Hungate tube at the end of trap 1 was interfering with the efficient recovery of radioactivity in the second trap. After removal of the tube, trap 2 was 100% efficient in trapping a known amount of $^{14}CO_2$. It is likely that the 10 mL dead space in the Hungate tube allowed the labelled gases to mix and not necessarily pass

through to the oxidation oven and onto the second trap. Consequently, increasing run times would increase the recovery of label because there was more time for the gases to flush through the Hungate tube. The Hungate tubes were removed from the system.

G.2 Effects of pressure on radioactivity counts obtained

One question that needed exploration was the potential effect of pressure changes in the microcosm headspace on the radioactivity counts obtained. In order to test this, 10 mL of air were added to each microcosm containing $H^{14}CO_3^-$ (acidified), and shaken vigorously. After equilibration for 1 h, 6 consecutive 1 mL samples were removed from the serum bottles. One mL of headspace was injected directly into fluor and then 1 mL was injected into the GC; this sequence was repeated three times. The samples were flushed for 5 min. Over 90% of the radioactivity was trapped in vial 1A, and decreasing headspace pressure had no effect on the counts obtained.

Comparison between the direct injection counts (1068 dpm) and the trapping counts (148 to 136 dpm) showed a significant loss of radioactivity when the sample was passed through the trapping system. Since radioactivity is generally only found in vial 1A and not in any of the other three vials, it was possible that the radioactivity was being lost while the sample passed through the GC. Measurement of the flow rate of helium at the injection port of the GC was 124 mL/min, whereas the flow rate at the end of the GC column was 80 mL/min, suggesting the possibility of a leak in the column. Due to the age of the column, a new column (10 ft x 3/16 inch) packed with Porapak R resin was prepared and installed in the GC. The flow rate at the injection port and the back end of the column were both 112 mL/min. Headspace samples were analyzed and the % recovery of the radioactivity (counts in trapping vials/counts from direct injection) increased to 60%.

In order to eliminate the GC as the potential problem for loss of radioactivity, the GC was by-passed and the helium flow was connected directly to the trapping system (via the GPC internal network). Injections of headspace gas of microcosms containing $H^{14}CO_3^-$ which had been acidified resulted in greater than 100% recovery of $^{14}CO_2$ in the trapping

vials compared to counts obtained by directly injecting headspace samples into fluor. As noted before, more than 90% of the radioactivity was trapped by the first vial (1A). Therefore, it was decided that the GC contributed to loss of radioactivity and should not be used as part of the detection system.

Once it was decided to by-pass the GC, the helium was permanently directed to the GPC unit and an injection port was created to access the trapping vials. Flow rate was measured at 70 mL/min at the injection port and after trap 1 but was 60 mL/min after trap 2.

G.3 Detection of $^{14}CH_4$ oxidized to $^{14}CO_2$

Having already found that $^{14}CO_2$ could be trapped efficiently in trap 1, it had to be determined whether the second trap was able to trap oxidized $^{14}CH_4$. To do this, $^{14}CH_4$ was generated *in situ* by the addition of ^{14}C-glucose to methanogenic microcosms containing sewage sludge. Twenty-five milliliters sewage sludge (obtained from Gold Bar Wastewater Treatment Plant) were added to 58-mL serum bottles after which 5.8 x 10^5 dpm ^{14}C-glucose was added. The ^{14}C-glucose stock was diluted with unlabelled glucose so that the microcosms had a final concentration of 140 mg/L glucose. Heat-killed sterile controls were also prepared. The microcosms were incubated in the dark at room temperature for 3 weeks before analysis of $^{14}CO_2$ and $^{14}CH_4$. One milliliter headspace sample was injected into the system and radioactivity was found only in the first trap (1A) with radioactivity counts close to those obtained from directly injecting sample into fluor. This was repeated with each microcosm, and no radioactivity was ever detected in the second trap.

These results suggested that either no $^{14}CH_4$ had been generated *in situ* or that the oven was not oxidizing $^{14}CH_4$ to $^{14}CO_2$. To confirm the presence of methane in the microcosms, total methane (labelled and unlabelled) was measured by GC and found to be 15 to 18% vol of the headspace. Glucose is easily oxidized under methanogenic conditions (Zinder 1993) and should yield equal amounts of CH_4 and CO_2 (according to Buswell's equation), therefore the generation of $^{14}CO_2$ without $^{14}CH_4$ was unlikely, and it was more probable that $^{14}CH_4$ was present but not being detected.

Using a temperature probe, the temperature of the oxidation oven was confirmed to be 800°C. Examination of the quartz oxidation tube containing copper oxide indicated that the copper oxide had become reduced. After replacement of the oxidation tube, low level radioactivity was detected in both traps: $^{14}CO_2$ traps (120 dpm) and $^{14}CH_4$ traps (70 dpm).

There was concern that the low amount of $^{14}CH_4$ being generated by the ^{14}C-glucose microcosms was insufficient to test the efficiency of the trapping system to oxidize and trap $^{14}CH_4$. Therefore, fresh microcosms were established with ^{14}C-2-acetate and sewage sludge. The methyl group of acetate is known to be preferentially reduced to CH_4 (Pine and Barker 1955), therefore using ^{14}C-2-acetate would ensure the generation of $^{14}CH_4$. Flushed 58-mL serum bottles (sterile) received 30 mL of recently collected sewage sludge and 1 mL of acetate stock for a total radioactivity addition of 4.2×10^5 dpm. After 30 d incubation, headspace samples were injected into the trapping system and $^{14}CH_4$ was successfully detected at higher counts than $^{14}CO_2$.

G.4 Problems with the oxidation tube

It was observed that the oxidizing tube was frequently becoming reduced. That is, the copper oxide was generally reduced and needed replacement each time the system was used. This suggested that large amounts of organics were being oxidized exceeding the amount of oxidation of $^{14}CH_4$ to $^{14}CO_2$. Extensive examination of the events leading up to the oxidation of the tube was conducted by addressing the following questions. 1. Can the oxidation tube be "recharged" (oxidized) by injecting air through the heated column? Yes, injection of 50 to 100 mL of air was sufficient to oxidize a heated column. 2. Does the helium flow alone through the system cause the oxidation tube to become reduced? No, after allowing the helium to flow through the oven for 4 h, no reduction of the tube occurred. 3. Does the helium flow through the empty trapping system cause the tube to become reduced? No, after 4 h of helium flow, the tube showed no signs of being reduced. 4. Does the helium flow through the trapping system containing fluor and Carbosorb cause the tube to become reduced? Yes, within minutes, the tube began to show signs of

reduction and after a couple hours the tube was completely reduced. The fluor contains xylene and methanol, both of which are volatile and are likely being oxidized in the oven, causing the copper to become reduced.

Since the fluor was contributing to a prematurely reduced column, there was a concern that it was interfering with the oxidation of $^{14}CH_4$ to $^{14}CO_2$, which would result in inaccurately low recoveries of $^{14}CH_4$. Since the mineralization of the various compounds being tested was likely to be low, the detection system needed to be accurate, efficient and sensitive. Thus it was decided to trap the $^{14}CO_2$ and oxidized $^{14}CH_4$ using KOH. With this design, the trapping vials were filled with 10 mL of 0.5 M KOH and 100 μL concentrated acetic acid (to minimize the chemiluminescent interference caused by KOH). After flushing, 1 mL of the KOH solution was added to 10 mL fluor and counted in the scintillation counter. The radioactivity counts obtained from the ^{14}C-glucose and ^{14}C-acetate microcosms were slightly higher when label was trapped in the KOH than when trapped directly in fluor, however the values were not reproducible.

G.5 Final modification: double sampling technique

Using the KOH trapping solution solved the problem of premature reducing of the oxidation column, however the method did not provide reproducible results and only measured 1/10 of the label originally trapped. An alternative modification was sought, in which trap 1 was isolated from the oven so the presence of fluor in this trap would have no effect on the oven. Injection of 1 mL headspace trapped $^{14}CO_2$ in the first set of vials after which a slight modification diverted the helium flow from trap 1 directly through the oven to trap 2. Injection of a second 1 mL headspace sample would travel through the oven (without fluor vapors) and into trap 2 containing fluor and Carbosorb where produced $^{14}CO_2$ and oxidized $^{14}CH_4$ would be trapped. The amount of $^{14}CH_4$ produced would be determined by subtracting the dpm values of the first trap (only produced $^{14}CO_2$) from the dpm values of the second trap (produced $^{14}CO_2$ plus oxidized $^{14}CH_4$). The flow rate of the He was decreased to 40 mL/min. Repeated sampling of the ^{14}C-glucose and ^{14}C-acetate

microcosms resulted in almost identical radioactivity counts (Table G1). This system was determined to provide reproducible results and did not cause premature reduction of the oxidation tube.

The suitability of the double sampling technique was confirmed when compared to the radioactivity counts obtained by the alternative methods tested (Table G1). The double sampling technique yielded reliable results and appeared to be more sensitive than the other methods tested. As well, it was the only method that produced the 1:1 ratio between $^{14}CO_2$ and $^{14}CH_4$ expected in the ^{14}C-glucose supplemented microcosms. The $^{14}CH_4$ generated *in situ* was detected using the double sampling method and therefore was considered reliable for detecting the production of $^{14}CO_2$ and $^{14}CH_4$ in methanogenic microcosms.

Table G1: Summary of the radioactivity counts obtained in trials using the alternative ^{14}C-trapping methods tested. Values (dpm) represent total activity in the microcosm headspace where total activity = dpm/mL x headspace volume (mL)

Method	Trial	^{14}C-acetate		^{14}C-glucose		
		CO_2 (dpm)	CH_4 (dpm)	CO_2 (dpm)	CH_4 (dpm)	CH_4/CO_2 ratio
Into ACS	1	864	0	5248	4160	0.79
(traps in series)	2	1080	0	5760	5920	1.02
	3	1026	2079	3744	1312	0.35
Into KOH	1	280	5040	5120	3520	0.68
(traps in series)	2	8120	12040	—[a]	—	—
	3	0	5130	—	—	—
	4	5130	2700	—	—	—
Double	1	1652	5236	3968	5130	1.29
Sampling	2	1960	5628	4288	4672	1.09
(traps isolated)	3	1708	5908	4928	4832	0.98

[a] test was not performed

Appendix H: Enumeration of H_2-utilizing methanogens

Table H1: Enumeration of H_2-utilizing methanogens in the fine tailings samples obtained from the MLSB in August 1997 and July 1998. Values are expressed as MPN/g dry weight except for the 1 m water samples which are MPN/mL.

Depth (m)	Aug 1997 MB1	Aug 1997 MB2	July 1998 MBC1	July 1998 MBC2
1	9.00×10^4	3.00×10^3	1.70×10^4	NS[a]
5	4.77×10^6	3.47×10^5	1.02×10^6	3.13×10^5
8	NS[a]	NS	2.07×10^5	NS
10	4.56×10^6	3.19×10^5	5.08×10^4	9.20×10^4
15	7.18×10^5	4.79×10^5	NS	1.44×10^5
20	2.58×10^6	1.05×10^5	NS	1.34×10^5

[a]site was not sampled

Table H2: Enumeration of H_2-utilizing methanogens in the fine tailings samples obtained from the Demonstration Pond and the Base Mine Lake in August 1997 and July 1998. Values are expressed as MPN/g dry weight except for the 1 m water samples which are MPN/mL.

Depth (m)	1997 DP	1998 DP	1997 MBIP1	1998 MBWIP
1	< 20	< 2	2.30×10^4	3.00×10^4
5	4.20×10^4	$> 4.37 \times 10^5$	NS[a]	NS
10	NS[a]	NS	2.86×10^{10}[b]	1.60×10^7

[a]site was not sampled

[b]second enumeration of this sample after 7 mon storage at 4°C produced an MPN value of 7.30×10^6/g.